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GENETIC AND FUNCTIONAL STUDIES OF LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A2 AND SECRETORY PHOSPHOLIPASE A2 VARIANTS: THEIR POTENTIAL ROLE IN ATHEROSCLEROSIS

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To Heather and my family.

Declaration

All of the work reported in this thesis is my own, or has been carried out as part of a collaboration in which I played a major part. All collaborative work has been acknowledged in the text. No part of this thesis has been submitted for a degree, diploma or other qualification at any other university.

Peter T E Wootton

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Publications resulting from this thesis

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Abstract

Phospholipase A2 (PLA2) enzymes are characterised by their ability to hydrolyse the sn-2 position of glycerophospholipids, generating lyso-phospholipids and free fatty acids (FFA) in significant concentrations. The aim of this thesis was to investigate the role of Lipoprotein-associated PLA2 (Lp-PLA2, *PLA2G7*) and two secretory PLA2 enzymes (IIA and V, *PLA2G2A* and *PLA2G5*) in the progression of atherosclerosis. All three enzymes may be implicated in the oxidative modification of lipoproteins, and the release/removal of powerful inflammatory mediators. The first objective of this thesis was to try and resolve the contribution of Lp-PLA2 to CHD. The relationship between statins and *PLA2G7* expression was also investigated. Secondly, the association of haplotypes in the *PLA2G2A* and *PLA2G5* genes with lipid markers and sPLA2-IIA mass levels was determined for the first time.

PLA2G7

Using SSCP analysis, the *PLA2G7* promoter was screened for novel polymorphisms. A G to A change was identified 1230 base pairs 5' of the transcription start site (rare allele frequency of 0.22), and between potential RORalpha and OCT1 transcription factor binding sites. The G-1230A and activity-altering A379V polymorphisms, were then investigated in several studies with regards to measures of Lp-PLA2 activity, LDL particle oxidation, CHD risk, body composition, and other intermediate phenotypes related to the enzyme's potential role in atherosclerosis. In the prospective NPHS II study, quartiles of Lp-PLA2 activity showed no association with CHD risk (p=0.37 after adjustment for age and sex). However, the 379V allele was associated with higher Lp-PLA2 activity (p=0.05) and specific activity (p=0.001). In the EPIC-Norfolk case-control study, higher Lp-PLA2 activity was associated with higher CHD risk (p<0.0001), although adjustment for LDL removed this association (p=0.45). In agreement with the NPHS II study, the 379V allele was associated with a higher Lp-PLA2 activity (p=0.03). In the UDAC study of Caucasian diabetic men and women, individuals with the metabolic syndrome (MS) were associated with higher Lp-PLA2 activity compared to those without (p=0.02). There was also an inverse relationship of Lp-PLA2 activity and oxLDL/LDL (p=0.03). There was no significant association of the A379V genotype with either Lp-PLA2 enzyme activity (p=0.34) or oxLDL/LDL levels (p=0.32). The A379V allele was not found to be associated with CHD risk in any of the investigated studies (p<0.06). Finally, the association of the A379V variant with body composition changes was investigated in a longitudinal study of 123 male Caucasians over 10 weeks of physical training (BH2 study). After exercise training, the 379V allele was associated with a decrease in percentage adipose tissue mass (p=0.01) and increase in

percentage lean mass (p=0.01) compared to the other genotype groups. The results presented in this thesis raise further questions about the causal relationship of the Lp-PLA2 enzyme with CHD and its suitability as a clinical marker of disease.

In parallel to these investigations, *in vitro* work was performed to determine whether the novel G-1230A variant influenced gene transcription. Human liver Huh-7 cells were transiently transfected with different G-1230A reporter constructs driven by the *PLA2G7* promoter, and assayed for differences in luciferase activity. No difference in activity was observed between the -1230 alleles over six experiments (p=0.94). In addition, the novel G-1230A SNP was not associated with enzyme activity (p=0.77) and oxLDL/LDL levels (p=0.07) in the UDAC study; as well as % adipose tissue and % lean mass (p=0.91 and p=0.92 respectively) in the BH2 study. The G-1230A variant was not associated with CHD risk (p>0.22 in all models) in the case-control HIFMECH study. Taken together, these *in vitro* and epidemiological analyses suggest that the novel G-1230A variant does not represent a putatively functional variant.

The previously observed association of lower Lp-PLA2 activity with statin use in UDACS (p=0.04) was further investigated *in vitro*. Human macrophages of known A379V genotype were exposed to Simvastatin for 48hrs. Overall there was a 17.6% and 27.2% reduction in gene expression (RT-PCR) when treated with 10μM and 25μM of Simvastatin, respectively (p<0.01). There was no heterogeneity of effect between A379 or 379V homozygotes (p=0.96). This reduction in expression suggests that the pleiotropic effects of statins may independently influence *PLA2G7* expression

PLA2G2A and PLA2G5

Using publicly available re-sequencing data, tagging SNPs (tSNP) and common haplotypes were identified in the UDAC study for the *PLA2G2A* and *PLA2G5* genes. Overall, haplotypic variation in the *PLA2G2A* gene was associated with significant effects on sPLA2 IIA mass levels (p<0.0001). The most frequently occurring haplotype was associated with 53% higher sPLA2 IIA levels compared to the five other most common haplotypes (p<0.00001). There was no significant association of any haplotype with other measured traits. In addition, three haplotypes in the *PLA2G5* gene were associated with raising effects on TG (p<0.01), cholesterol (p<0.001) and LDL (p<0.01) levels compared to the most common haplotype. The rarest haplotype was also associated with a lower oxLDL/LDL level (p=0.01). This study represents the first investigation of genetic variation in the *PLA2G2A* and *PLA2G5* genes with regards to atherosclerotic risk traits and sPLA2-IIA levels.

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Abbreviations commonly used

AA	Arachidonic acid
ANOVA	Analysis of variance
ANCOVA	Analysis of co-variance
Apo	Apolipoprotein
BH2	Bassingbourn Army study II
BMI	Body mass index
bp	Base pairs
CHD	Coronary Heart Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid disodium salt
EPIC-Norfolk	The European Prospective Investigation of Cancer, Norfolk
FA	Fatty acid
FBS	Foetal Bovine Serum
FFA	Free fatty acid
HD	Heteroduplex
HDL	High Density Lipoprotein
HIFMECH	Hypercoagulability and Impared Fibrinolytic function MECHanisms
HR	Hazard ratio
HW	Hardy Weinberg
ICAM	Inter-cellular adhesion molecule
IDL	Intermediate density lipoprotein
IL	Interleukin
kb	Kilobase
kDa	Kilo dalton
Km	Michaelis constant
LDL	Low density lipoprotein
LPA	Lyso-Phosphatidic acid
Lp-PLA2	Lipoprotein associated phospholipase A2
mRNA	Messenger ribonucleic acid
MS	Metabolic syndrome

NPHS II	The second Northwick park hospital study
nt	Nucleotides
oligo	Oligonucleotide
oxLDL	Oxidised LDL
oxLDL/LDL	Oxidised LDL/LDL
PAA	Polyacrylamide
PAF	Platelet activating factor
PAFAH	Platelet activating factor acetylhydrolase
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PLA2	Phospholipase A2
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
sdLDL	Small dense LDL
SDM	Site directed mutagenesis
SEM	Standard error of the mean
sPLA2	Secretory Phospholipase A2
SSCP	Single strand conformation polymorphism
TAOS	Total anti-oxidant status
TBS	Tris borate solution
TEMED	N,N,N',N'-tetraethylethylenediamine
TG	Triglycerides
TNF	Tumour necrosis factor
UDACS	University college diabetes and cardiovascular study
UKPDS	The UK Prospective Diabetes Study
UTR	Untranslated region
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
Vmax	Maximal velocity (maximum rate of reaction)
WHO	World health organisation
WT	wildtype

CHAPTER 1

INTRODUCTION

1.1 The global burden of atherosclerosis

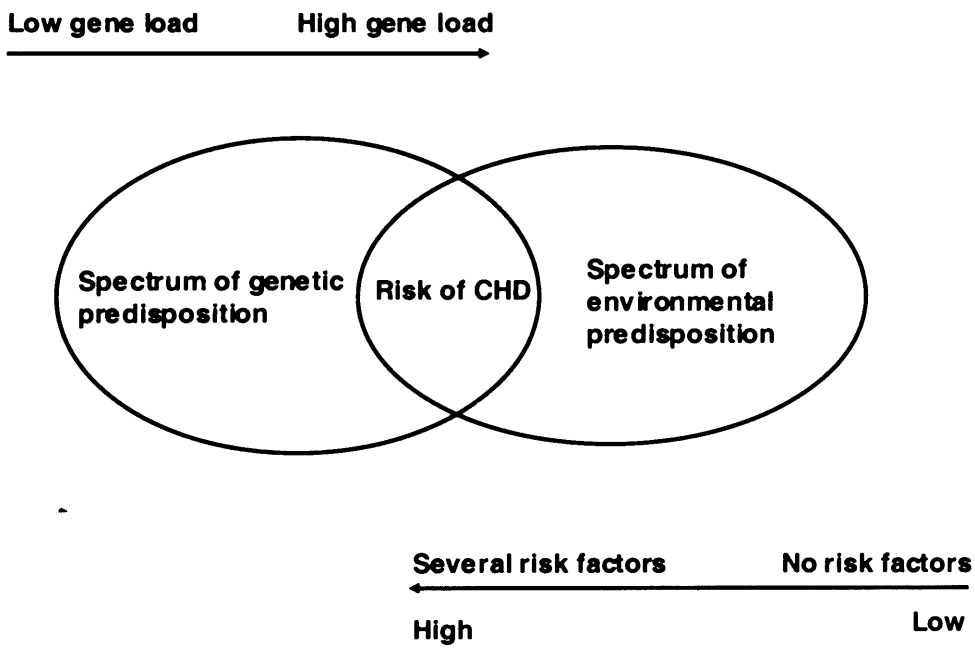
Despite huge advances in therapy, care, and lifestyle changes, Coronary Heart Disease (CHD) remains the leading cause of death in the developed countries of North America, Europe, and much of Asia. CHD is the single most common cause of death in America, with a mortality rate of one in every 4.9 deaths, suggesting a continuing emergence of pandemic proportions (Breslow, 1997; Braunwald, 1997). Atherosclerosis itself is a multi-factorial disease involving a close interaction between genes and the environment (Talmud and Humphries, 2002). Prospective epidemiological approaches have identified several 'risk factors' such as sex, age, poor diet, lack of exercise, smoking, hypertension, diabetes mellitus and hyperlipidemia (McGovern et al., 1996; Gensini et al., 1998b). Some of these risk factors involve strong genetic components (of which familial predisposition is an indicator) which further contribute to this condition (Marenberg et al., 1994b).

This thesis has concentrated on three particular Phospholipase A2 enzymes which are closely linked through their specific activities with the oxidative modification of lipoproteins and inflammation; processes widely regarded as important in the generation of an atherosclerotic plaque (Ross, 1999).

1.2 Risk factors for Atherosclerosis

The concept of cardiovascular risk has evolved from epidemiological studies of CHD established in the 1940s and 50s such as the Framingham study (Kannel, 2000). Using a prospective epidemiological approach, it has been possible to demonstrate a consistent association of characteristics observed in apparently healthy individuals with the subsequent development of CHD. Measurable traits that are significantly associated with disease do not in themselves provide proof of causality or establish a pathophysiological relationship. However, they do further the understanding of how atherosclerosis manifests itself. With the formal completion of the human genome project (Lander et al., 2001), a wealth of information has become available, in particular, the variation of the human genome (Sachidanandam et al., 2001) and its relationship to atherosclerosis and other diseases that involve complex gene-gene and gene-environment interactions. In a general population there is a range of genetic risk profiles, with each individual occupying a position along the genetic risk spectrum. This is contrasted with an environmental spectrum dependent on a range of lifestyle choices (e.g. smoking or diet). It is therefore conceivable to see that when an individual enters a high risk environment with regards to CHD and has a high genetic risk, then premature disease could develop (Fig.1.1). In effect, the analogy that could be applied would be that of a gun, with genetics providing a 'loaded gun', and the environment being the 'trigger' (Olden and White, 2005).

Fig. 1.1: Potential gene environment interaction in the progression of CHD



This first section of this chapter aims to highlight the more commonly acknowledged risk factors, some of which are influenced by genetics, that combine over time leading to an observed increase in CHD risk. Indeed, it has been shown consistently that the risk of CHD increases markedly with age (itself regarded as a risk factor) in both men and women possibly as a result of this accumulation of risk factors (Stamler et al., 1999).

1.2.1 Environmental factors

1.2.1.1 Diet

The process of atherosclerosis involves the accumulation of cholesterol, inflammatory cells, fibrous tissue and calcium in the arteries. The build up is intensified through an excess of cholesterol and triglycerides which can result from a diet high in fats, particularly saturated fats, and dietary cholesterol (Schonfeld et al., 1982; Kritchevsky, 1999). This contrasts with mono- and poly- unsaturated fats which lower Low density lipoprotein-cholesterol (LDL-C) (Kris-Etherton et al., 1999). High fat diets have been shown to be associated with risk of CHD [reviewed in (Assmann et al., 1999)]. Excess sodium salt in the diet can also lead to adverse changes in blood pressure through the retention of Sodium chloride in the kidneys, and increase the likelihood of a cardiovascular event (Tobian, 1997).

1.2.1.2 Smoking

Cigarette smoking has been defined as a major independent risk factor that doubles life-time risk of CHD, with synergistic effects when combined with other cardiovascular risk factors (Doll and Hill, 1966; Gensini et al., 1998; Weintraub, 1990). However, cessation of smoking rapidly decreases risk of CHD (Gensini et al., 1998). Several mechanisms are thought to relate to this increase in risk. Smokers have a raised pro-coagulant state that increases the risk of arterial thrombosis (Meade et al., 1986). Products of tobacco smoke lead to increased endothelial dysfunction (an important pre-requisite for the development of an atherosclerotic plaque), and an up-regulation in adhesion molecules which are required for the attachment of monocytes and platelets (Lu and Creager, 2004). Furthermore, smoking disrupts lipoprotein metabolism by increasing insulin resistance and lipid intolerance, and leads to the production of small dense LDL (Eliasson et al., 1997). By stimulating catecholamines, smoking up-regulates hormone sensitive lipase, increasing circulating free fatty acid levels (Eliasson et al., 1997), therefore causing atherogenic dyslipidaemia. Smokers are thought to have lower levels of anti-oxidants, supporting the hypothesis that smoking favours the oxidation of LDL (Fickl et al., 1996).

1.2.1.3 Lack of Exercise

It is hard to assess whether lack of exercise itself represents an independent risk factor. However, regular leisure time physical activity has been shown to lower the risk of CHD (Wannamethee and Shaper, 2001), and favourably affects blood pressure, weight, glucose tolerance, HDL-cholesterol, and triglyceride levels (Hardman, 1996).

1.2.1.4 Other potential environmental risk factors

Along with the three established 'lifestyle' risk factors, several other potential environmental risk factors have been identified. Whether these are important in the development of atherosclerosis is still under debate, and could simply be innocent by-standers to other environmental impacts. Air pollution (Liao et al., 2005) and infection (namely from *Chlamydia pneumoniae*) (Blasi, 2004) leading to a chronic inflammatory state are both potentially related to the progression of atherosclerosis. In addition, the pathologic and nutritional status of a mother during foetal development could lead to the increased progression of disease in off-spring by as yet unknown mechanisms (Lusis, 2003).

1.2.2 Factors with a strong genetic component

Atherosclerosis is a complex disease taking many years to present and involving a wide array of biological processes. It is likely that in different individuals in the population, variation within the genes of multiple implicated mechanisms will contribute to the heritability seen and overall genetic aspect of the disease. In support of this, large scale investigations into the many 'candidate genes' and genetic loci involved in processes related to atherosclerosis, such as; inflammation, lipid metabolism, thrombosis and cell development, has produced a wealth of association data too large to summarise in this chapter. However, some of the more studied genetic aspects of this disease have been discussed below.

1.2.2.1 Family History

Family history reflects a combination of both shared genetic and environmental factors, and is regarded as a classical independent risk factor for CHD (Goldbourt and Neufeld, 1986). That the risk associated with family history is not wholly explained by classical risk factors, supports the involvement of a strong genetic component (Hawe et al., 2003). Genetic factors are of particular importance in the aetiology of early onset disease events, as shown in a study of monozygotic and dizygotic twins, where death from CHD was found to be influenced by genetic factors in both men and women (Marenberg et al., 1994a). In addition, most early CHD events (below the age of 40) have been reported to occur in families with a positive history of CHD, in particular those individuals with a history of CHD in first degree relatives below the age of 65 (Pohjola-Sintonen et al., 1998).

1.2.2.2 Gender

Gender is an important predictor of CHD risk in those individuals under the age of 60, with men developing CHD at twice the rate of women (Hochner-Celnikier et al., 2002). Hormonal factors play an important role; the difference in risk between genders diminishes markedly after menopause, and oestrogen-replacement therapy reduces CHD risk by approximately 50% (Kafonek, 1994), although recent reports have questioned this effect (Fenton, 2003; Armitage et al., 2003).

1.2.2.3 Lipid metabolism

The most significant risk factors for CHD are related to lipoprotein metabolism (Lusis et al., 2004). Lipids are transported through the bloodstream complexed to proteins (apolipoproteins) which themselves are involved in the packaging and secretion of

lipids from cells, in the processing of circulating lipoproteins, and as ligands for cellular receptors. The apolipoproteins include apo (a) AI, AII, AIV, AV, B48, B100, CI, CII, CIII, and E. In addition to apolipoproteins, there are numerous enzymes involved in the transfer of lipids between lipoprotein particles and the lipolysis of particles, as well as several cellular receptors and ligands involved in metabolism and clearance. Low Density Lipoprotein (LDL) is the major cholesterol carrier in the blood, whereas High Density Lipoprotein (HDL) transports excess cholesterol back to the liver where it can be excreted with bile. The association of high levels of LDL-C, and low levels of HDL-C with CHD risk have previously been demonstrated in several studies (Tanaga et al., 2002; Coresh et al., 1993; Barter and Rye, 1996). Therefore, all of the genes (proteins) associated with HDL and LDL must be considered 'candidate genes' with the potential to alter the balance of lipid metabolism and consequently CHD risk. The shear number of candidate genes involved in this process means that it is very hard to give a comprehensive review of all the genes found to be associated with CHD risk. Listed below are a few selected examples.

The meta-analysis of recent epidemiological studies identified an association of raised levels of Lipoprotein (a) (Lp(a)) with an increased risk of CHD (Danesh et al., 2000). Lp(a) itself is a variant of LDL, with a cholesterol ester-rich core surrounded by a single ApoB100 molecule, but it also contains an additional polypeptide, termed apo(a), that is disulphide-bridged to ApoB. Lp(a) is a factor whose levels are remarkably stable within an individual over time, with 90% of the variation in Lp(a) concentrations being determined by genetic variation in the apolipoprotein a *(APOA)* locus (Boerwinkle et al., 1992). The structural similarity of Lp(a) to both the fibrinolytic enzyme plasminogen, and LDL, has suggested a pro-thrombotic and atherogenic role for this lipoprotein, possibly through its ability to impair fibrinolysis (Loscalzo et al., 1990).

Apolipoprotein E (ApoE) by contrast, has a protective role in atherosclerosis by maintaining overall plasma cholesterol homeostasis through the hepatic clearing of cholesterol-rich VLDL and Chylomicron remnants. ApoE also enables cellular cholesterol efflux from macrophage foam cells present within atherosclerotic lesions; and modifies both macrophage and T-lymphocyte mediated immune responses (Curtiss and Boisvert, 2000). The gene coding for apoE *(APOE)* has been extensively studied, with three common alleles (the most common being *ε3*, and the two variants *ε2* and *ε4*) coding for three major isoforms in plasma (Davignon et al., 1988). These three common variations of apoE lead to marked effects on plasma cholesterol levels: carriers of the *ε2* gene variant have on average cholesterol levels 10% lower than *ε3*

homozygous individuals, while *ε4* carriers have average cholesterol levels 5% higher than *ε3* homozygous individuals (Davignon et al., 1988). This in turn leads to a modest ε2-lowering and ε4-raising impact on CHD risk such that this genotype alone may explain 5-8% of the attributable risk of CHD in the population (Davignon et al., 1988).

Genetic variations in a number of other genes involved in lipid metabolism have also been shown to exhibit an effect on lipid levels and CHD risk. Genetic variation in the apolipoprotein-AI/CIII/AIV and recently described apolipoprotein AV gene cluster have been found to be associated with triglyceride (TG) levels (Pennacchio et al., 2001; Talmud et al., 2002a). Similarly, variation in the Cholesteryl Ester Transfer Protein (CETP) gene has been implicated in altering the activity of CETP. In particular, the TaqIB variant (intron 1) was found to be significantly associated with both plasma levels of HDL and also CHD risk in a large meta-analysis of 7 population studies (Boekholdt et al., 2005b). The association of the TaqIB variant with HDL and risk may be the result of Linkage Disequilibrium (LD), with recent work identifying several functional promoter polymorphisms that interact with each other and have a significant effect on CETP and HDL levels (Frisdal et al., 2005).

Triglycerides themselves are regarded as an independent risk factor (Hokanson and Austin, 1996), therefore, genetic variation in lipoprotein lipase which hydrolyses triglycerides into free fatty acids and glycerol, has also been considered as an important candidate gene. Mutations in this gene have been found to be associated with triglyceride levels, with one particular mutation (D9N) showing a strong gene-environment interaction with smoking (Talmud et al., 2002b). A final example is that of the LDL-receptor, where numerous mutations have been found associated with the disease Familial Hyperlipidemia (FH) (Austin et al., 2004). The disease itself exhibits a dominant mode of inheritance and the heterogeneity of receptor mutations makes molecular diagnosis of the disorder difficult. However, the disease is associated with dramatically elevated cholesterol levels and higher risk of CHD (Lusis et al., 2004).

1.2.2.4 Hypertension

Risk of CHD is well known to positively correlate with increasing blood pressure, and the risks of elevated systolic and diastolic blood pressure are additive (Stokes, III et al., 1989; Borghi et al., 2003). Clinical trials have also successfully demonstrated that intensive lowering of blood pressure reduces the risk of CHD (Hansson et al., 1998). Raised blood pressure increases the likelihood of endothelial damage to blood vessels, which may in turn lead to platelet aggregation, proliferation of vascular smooth muscle

cells, and reduced clearance of lipoproteins from the vessel wall (Himmelmann et al., 1998).

The genetic factors contributing to common forms of hypertension are largely unknown, with many potential candidates having small effects. As such, clinical hypertension may be the result of a number of gene-gene and gene-environment interactions. Association studies have suggested the involvement of genes relating to sodium epithelial channels, catecholaminergic/adrenergic function, and others involving lipoprotein metabolism, hormone receptors, and growth factors [reviewed in (Timberlake et al., 2001)]. Several gene candidates involved in the renin-angiotensin system (RAS) have been studied due to the observed effects of the RAS pathway on vasodilation and vasoconstriction of blood vessels. A polymorphism in the angiotensin II type 1 receptor gene (-535C>T) has shown association with hypertension. This polymorphism also showed a synergistic effect on risk of hypertension with the angiotensin I converting enzyme (ACE) insertion/ deletion (I/D) polymorphism. The combined effect seen may be related to an increased formation of angiotensin II, which is known to be a powerful vascular constrictor (Takahashi et al., 2000). In contrast, kinins are powerful vasodilators in the RAS pathway, and an insertion/deletion polymorphism in the kinin B1/B2 receptor (BK 1/2R) has shown association with risk of CHD related to hypertension (Dhamrait et al., 2003).

1.2.2.5 Diabetes and Glucose

By the year 2010, the number of people in the world with diabetes will be around 221 million (Amos et al., 1997; Orchard, 1998), rising to 300 million by the year 2025 (King et al., 1998). Patients with diabetes have a two to threefold higher incidence of CHD (Garcia et al., 1974), and those who present in the fourth and fifth decade have a twofold increase in mortality (Panzram, 1987). Diabetes remains one of the major risk factors as determined by the large prospective Framingham study (Castelli et al., 1986), and the importance of diabetes as a CHD risk factor has been demonstrated by the Multiple Risk Factor Intervention Trial (MRFIT), which examined the effects of three major risk factors (hypertension, smoking, and hypercholesterolemia)(Stamler et al., 1993). This study showed that patients with type 2 diabetes mellitus (T2DM) who are non-smokers, normotensive and have normal serum cholesterol, have the same risk from CHD as a non-diabetic subject who has two of these three risk factors present.

However, at the molecular level, diabetes is not one, but several diseases with a common phenotype. As such, this makes genetic studies into diabetes hard to

undertake since the definition of disease can be very different between two populations. In addition, by grouping several molecular disorders under one roof, there is a subsequent loss in power of the study. The major risk factors associated with the excess of CHD in those with diabetes include; hyperglycaemia, insulin resistance, dyslipidaemia, hypertension, obesity, smoking, albuminuria, and a pro-coagulant state. Optimisation of glycemic control, weight reduction, lowering of elevated blood pressure, correction of lipid abnormalities, and the cessation of smoking in diabetes can therefore lower the risk of developing CHD (Pyorala et al., 1997; Goldberg et al., 1998; UK Prospective Diabetes Study Group, 1998). Many of the risk factors and biological processes associated with atherosclerosis are also shared with diabetes, leading to diabetes being defined as a CHD risk factor in its own right.

Several monogenic forms of diabetes exist that can be divided into maturity-onset diabetes of the young (MODY), syndromes of insulin resistance and mitochondrial diabetes (Barroso, 2005). MODY is recognised as an autosomal dominant disease with a variable phenotype, suggesting the influence of different genetic factors. Through the use of large scale linkage analysis, several genes have been implicated such as Glukokinase (Froguel et al., 1992), hepatocyte nuclear factors (Yamagata et al., 1996), and IPF1 transcription factors (Stoffers et al., 1997). Most of the genes identified are important factors in pancreatic β-cell (insulin secretors) development and function. Many other MODY genes are still to be identified, as between 16-45% of all MODY cases do not have mutations in currently identified genes (Velho and Robert, 2002). Insulin resistance syndromes are also a very heterogeneous group of disorders affecting the resistance of muscle, adipose tissue, and liver to the metabolic effects of insulin. Many of the genetic influences on this type of disorder comes from mutations present in the insulin receptor, but also important transcription factors involved in fatty acid metabolism such as the Peroxisome Proliferator-Activated Receptor gamma, PPARγ (Barroso, 2005). Mutations in mitochondrial DNA have also been associated with diabetes and are universally characterised as Wolfram syndrome in affected individuals (Barroso, 2005).

The monogenic forms of diabetes have also been informative with regards to polymorphism associations with 'late-onset' T2DM. For instance, PPARγ variants such as the Pro12Ala polymorphism have been consistently shown to be associated with T2DM (Altshuler et al., 2000), while genetic variation in the related PPARα gene has also been found to be associated with the age of onset and progression of T2DM (Flavell et al., 2005). In our laboratory we have found an association between the D

allele of the ACE gene and T2DM in a Caucasian population (Stephens et al., 2005). The common G-174C polymorphism in the IL-6 gene has also been found to be associated with both BMI and T2DM (Stephens et al., 2004; Vozarova et al., 2003). However, the very nature of the phenotype along with several environmental stimuli means that numerous genes (and their relevant polymorphisms) involved with T2DM have been characterised, some of which show contradictory results in different samples [reviewed in (Barroso, 2005)].

1.2.2.6 Inflammatory markers of atherosclerosis

Inflammation plays a crucial part in the initiation and progression of atherosclerosis and atherosclerotic events. Indeed, the recruitment of inflammatory cells such as monocyte-derived macrophages and T-lymphocytes to the intima of an artery at an early stage seems to support this hypothesis (Mullenix et al., 2005). Epidemiological and clinical studies have shown a strong and consistent relationship between markers of inflammation and CHD events (Ridker et al., 1997; Kuller et al., 1996; Ridker et al., 1998). As a result of this, measuring inflammatory markers may be useful for identifying individuals at a greater risk of having a CHD event. There is also the possibility that these markers could be involved in the progression of disease, and are potential targets for therapeutic agents. Statins main role is the lowering of LDL, however, patients with sometimes near-normal levels of LDL also have a reduced cardiovascular morbidity and mortality when taking a statin (Heart protection Study Collaborative group, 2002; The Scandinavian Simvastatin Survival Study group, 1994; Sacks et al., 1996). This reduction could be the result of the pleiotropic effects of statins, one of which is the 'dampening down' of inflammation (Steffens and Mach, 2004). Many potential inflammatory markers of CHD are under current investigation (Table 1.1), and recent attention has been focussed towards C-reactive protein (CRP) and the Phospholipase A2 enzymes (in particular, Lipoprotein-associated Phospholipase A2, Lp-PLA2) as promising markers of inflammation. This thesis has investigated the potential contribution of PLA2 enzymes to atherosclerosis. Lp-PLA2 in particular is seen as a robust marker, independent of other inflammatory markers and risk factors (Ballantyne et al., 2004; Blake et al., 2001; Blankenberg et al., 2003; Caslake et al., 2000; Oei et al., 2005; Packard et al., 2000b; Winkler et al., 2005). In addition, analysis conducted in the prospective ARIC study of 12,819 men and women showed that CRP and Lp-PLA2 levels have additive effects on the risk of CHD (Ballantyne et al., 2004).

CRP is an acute-phase reactant produced in response to acute injury, infection or other inflammatory stimuli, through a pathway regulated by IL-6 (Mullenix et al., 2005). CRP

mediates a variety of pro-inflammatory processes including activation of complement, immune cell chemotaxis, and platelet stimulation (Mullenix et al., 2005). CRP is also able to up regulate the expression of endothelial adhesion molecules such as ICAM and VCAM (Pasceri et al., 2001), as well as mediating the uptake of LDL by macrophages (Zwaka et al., 2001), both of which are important processes in the progression of atherosclerosis. CRP levels measured in several different populations, has shown an independent association with CHD risk even after adjusting for other potentially confounding risk factors (Albert et al., 2003; Koenig et al., 1999; Danesh et al., 2004). CRP levels also add predictive value in determining CHD risk when combined with LDL measures (Ridker et al., 2002).

Despite the overwhelming evidence that CRP is an excellent marker of CHD risk, doubts still remain to its influence on atherosclerosis. CRP could itself be causal; a confounder that simply marks the progression of disease; or it could represent an adaptive response to disease (reverse-causation). Under normal circumstances, a randomised drug (specific to CRP) intervention trial would establish whether CRP is indeed causal. Since no CRP lowering drug exists, an assumption that alleles are assigned randomly at birth (mendelian-randomisation) thereby preventing bias or confounding has been used to investigate causality (Shah *et al.*, 2005 in press). Several SNPs in the CRP gene promoter have been found to be associated with changes in CRP levels (Carlson et al., 2005), and a polymorphism (+1444C>T) in the 3'-untranslated region (UTR) has been found to influence CRP response following a mild inflammatory stimulus of periodontal therapy, the +1444T allele being associated with a higher level of CRP (D'Aiuto et al., 2005). Novel statistical approaches have been used to compare risk estimates for coronary events from genetic (CRP raising +1444C>T allele) and non-genetic association studies (CRP levels). Although the +1444T>C allele was associated with higher levels of CRP, it was not associated with risk, suggesting that CRP may not play an active role in atherosclerosis (Shah *et al.*, 2005 in press). If a causal relationship between CRP genotype and CRP levels were clearly established, then an association between CRP genotype and CHD risk would have provided indirect evidence for the causality of the association between CRP levels and CHD risk.

Table 1.1: Circulating markers of atherosclerotic inflammation under investigation (Taken from (Mullenix et al., 2005)).

Putative Markers	Abbreviation
C-reactive protein	CRP
Oxidised LDL	OxLDL
Phospholipase A2	PLA2
Platelet activating inhibitor-1	PAI-1
Homocysteine	-
Interleukin 1,2,6,7,8,18	IL-1,2,6,7,8,18
Myeloperoxidase	-
Tumour necrosis factor –α	TNF- α
D-dimer	-
Interferon-α	IFN- α
Monocyte chemotactic protein-1,-4	MCP-1,4
CD-40 Ligand	CD-40L
Parathyroid hormone-related protein	PTHrP
Matrix metalloproteinase- 9	MMP-9
Endothelin-1	-
Angiotensin II	AngII
Fibrinogen	-
Serum amyloid- A	SAA
Transforming growth factor- β	TGF- β
Intracellular adhesion molecule	ICAM
Lipoprotein (a)	Lp(a)
Leukotrienes	-
Peroxisome proliferator-activated receptors	PPAR

1.2.2.7 Haematological factors and coagulation

Thrombosis, triggered by atherosclerotic plaque rupture, is the ultimate and decisive cause of CHD-associated morbidity and mortality, with non-occlusive thrombosis also playing a role in lesion enlargement and vessel stenosis. An impaired fibrinolytic-coagulation system, such as increased concentrations of inhibitors of fibrinolysis or activators of coagulation can increase the risk of CHD. Clot formation relies on two pathways, the extrinsic and intrinsic pathway. Both pathways involve a number of coagulation factors, many of which are associated with risk of CHD.

Genetic variation in the coagulation system (fibrinogen, prothrombin, FV Leiden, FVII, and FXIII), and fibrinolytic pathway (PAI-1, and thrombin-activatable fibrinolysis inhibitor) have been studied with regards to CHD and ischemic stroke [reviewed in (Voetsch and Loscalzo, 2004)]. An example of these studies is that of fibrinogen itself. High levels of plasma fibrinogen lead to an increased likelihood of blood clots forming and constitute an independent risk factor for CHD (Heinrich et al., 1994; Eriksson et al., 1999). However, attempts to identify common variants influencing fibrinogen gene expression have brought conflicting results. Both non-synonymous and promoter polymorphisms have been identified in the three separate genes that code for the α, β and γ chains of fibrinogen. The heritability of fibrinogen is relatively low (25-30%), it therefore seems unlikely that polymorphisms would greatly influence its plasma concentration, although changes in the coding region could affect function (Franco and Reitsma, 2001). The T312A variant within the α chain is thought to influence clot stability (Standeven et al., 2003), and a study has shown that the 312A allele was associated with decreased survival after ischemic stroke (Carter et al., 1999). However, with no meta-analysis of current data, and other studies disputing these findings, the exact role of the T312A polymorphism (and other fibrinogen gene variants) in the thrombotic risk of CHD remains to be clarified.

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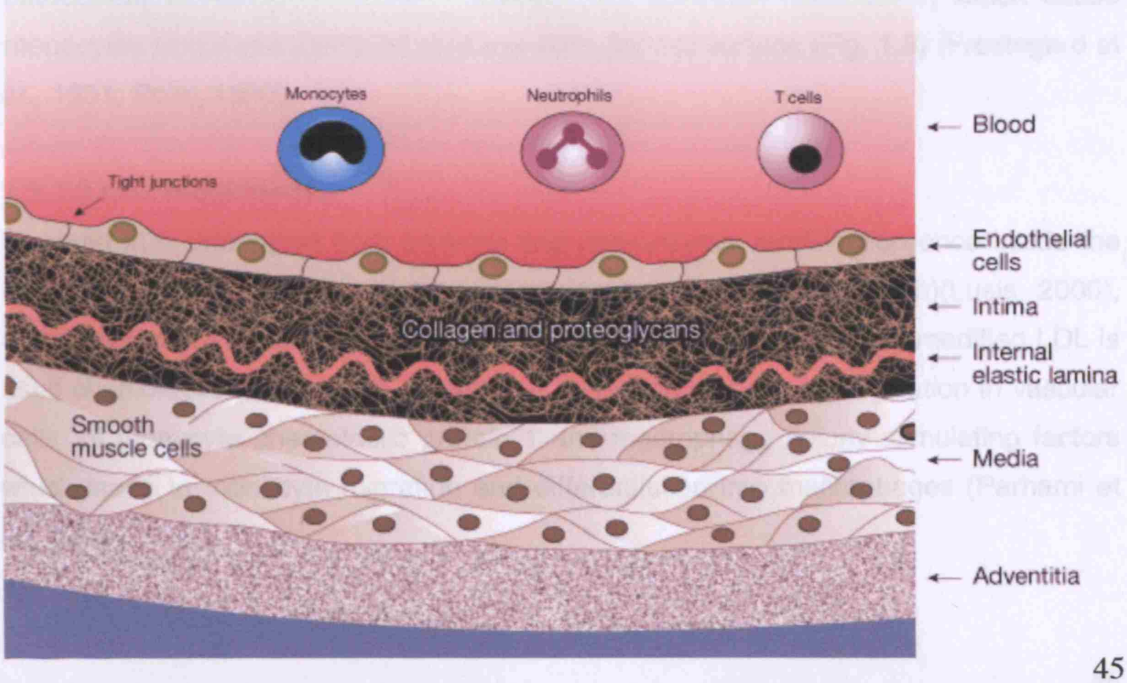
1.2.3 Definition of the metabolic syndrome

The concept of the metabolic syndrome (MS) has existed for over 80 years, with various definitions relating to metabolic disturbances that are themselves risk factors for CHD and already described in this introduction (Eckel et al., 2005). Several studies have found that the MS is associated with an increased risk of both Diabetes (Grundy et al., 2004) and CHD (Isomaa et al., 2001; Lakka et al., 2002). The definition of the metabolic syndrome has in some ways been standardised by the introduction of a World Health Organisation (WHO) classification (Alberti and Zimmet, 1998). Subsequent criteria have been applied by the National Cholesterol Education Program's Adult Treatment Panel III (NCEP:ATP III) (The NCEP panel, 2001), and the European Group for the Study of Insulin resistance (Balkau and Charles, 1999). Most of the current definitions involve several of the following conditions: Insulin resistance, obesity (usually measured by Body Mass Index, BMI), dyslipidaemia, hypertension, and lowered glucose tolerance (Eckel et al., 2005). Insulin resistance is widely accepted as being the unifying hypothesis with regards to the pathophysiology of the MS (Eckel et al., 2005). Due to the differences in these definitions it is hard to estimate the prevalence of the MS, however, recent reviews using the ATP III guidelines has shown variation in urban populations (worldwide) of 8-24% in men, and 7-43% in women over the age of 25 (Cameron et al., 2004).

1.3 Pathological development of cardiovascular disease

A normal large artery consists of three morphologically distinct layers: the intima, the innermost layer, is bound by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, on the peripheral side. The normal intima is a very thin region and consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, the middle layer, consists of Smooth Muscle Cells (SMC). The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs (Fig. 1.2) (Lusis, 2000). In the 19th century there were two competing hypotheses for the pathogenesis of atherosclerosis; the 'incrustation theory' proposed by the pathologist Von Rokitansky, suggesting intimal thickening resulting from fibrin deposition in the arterial wall; and the 'lipid infiltration' theory proposed by the pathologist Virchow, which suggested that lipid infiltration into the arterial wall followed by complex formation with mucopolysaccharides, was the initial event in atherosclerosis (Karsch, 1992). In 1973 Ross and Glomset first proposed the 'response to injury' hypothesis (Ross and Glomset, 1973; Ross and Glomset, 1976b; Ross and Glomset, 1976a). The most recent response to injury hypothesis describes repeated cycles of vascular endothelial injury, followed by repair (Ross, 1999). This mechanism also relies on certain pathological conditions such as inflammation and lipid ingress, to progress from a fatty streak during childhood, to an unstable lesion in adult hood.

Figure 1.2: Schematic of a Large Normal Artery in humans. Atherogenic events are usually initiated at sites of disturbed blood flow, where endothelial cells have no particular orientation and as such have increased permeability (Taken from (Lusis, 2000))



1.3.1 The stepwise progression of an Atherosclerotic Plaque

1.3.1.1 Initiation

The first step in the development of a plaque is repeated haemodynamic and chemical damage to the luminal vessel wall leading to endothelial dysfunction. Possible causes of endothelial dysfunction are elevated and modified LDL; free radicals caused by cigarette smoking, hypertension, and diabetes mellitus; genetic alterations; elevated homocysteine concentrations; and infections by micro-organisms (Ross, 1999). As discussed previously, the complexity of atherosclerosis is at least partly attributable to the various combinations of risk factors that may lead to dysfunction. Endothelial cells in this context represent a selectively permeable barrier between blood and intima; and have sensory and executive functions that control thrombosis, inflammation, vascular tone / remodelling (Lusis, 2000).

1.3.1.2 Accumulation of LDL and Leukocyte adhesion

A primary point in atherogenesis is the accumulation of LDL within the sub-endothelial matrix, which is exacerbated when levels of circulating LDL-C are high. LDL and other ApoB lipoproteins diffuse passively through endothelial cell (EC) junctions, and are retained in the vessel wall by an apparent interaction between ApoB-100 and matrix proteoglycans (Lusis, 2000). The process of increased permeability is mediated by cell signalling molecules such as; Nitric Oxide, Prostacyclin, platelet-derived growth factor, angiotensin II, and endothelin. A second critical step is the inflammatory-mediated up-regulation of leukocyte adhesion molecules (L-selectin, Integrins, Platelet-endothelial-cell adhesion molecule 1) and endothelial adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule 1, vascular cell adhesion molecule 1) which cause monocytes to roll and then bind to the endothelial cell surface (Fig. 1.3) (Frostegard et al., 1991; Ross, 1999).

1.3.1.3 LDL modification

Trapped LDL undergoes both enzymic and non-enzymic modification once inside the oxidative environment of the intima (oxidation, aggregation and fusion)(Lusis, 2000), and is described in further detail in section 1.3.3. So called, minimally modified LDL is itself chemotactic (Quinn et al., 1988), but it also induces the up-regulation in vascular cells, of monocyte chemotactic protein 1 and macrophage colony stimulating factors which leads to monocyte migration and differentiation into macrophages (Parhami et al., 1993).

1.3.1.4 Inflammation and Foam cell formation

The presence of oxidised LDL within the arterial intima can prevent the egress of monocytes out of the arterial wall stimulating further recruitment (Yla-Herttuala et al., 1989). Unlike the normal uptake of LDL by LDL receptors on macrophages, scavenger receptor uptake of oxLDL is not subject to any negative feedback, leading to a massive uptake of Cholesterol by differentiated macrophages (Khoo et al., 1992). The significant generation of foam cells (mediated by macrophage colony-stimulating factor, TNF α, and IL-1) which are laden with cholesterol in vacuoles, appear at a microscopic level as a fatty streak. There is also migration of smooth muscle cells and T-cells which further exacerbates this inflammatory response (Fig. 1.3)(Lusis, 2000; Ross, 1999).

1.3.1.5 Plaque maturation and remodelling

There is a conventional concept that these fatty streaks grow inexorably over years, accumulating lipid until they impede blood flow, leading to clinical symptoms. However, there is a process of re-modelling: the artery is able to expand outwards, accommodating the plaque without decreasing the lumen diameter. There is a limit in plaque size after which the lumen is narrowed and the patient may then present with the classical clinical symptoms of stable angina pectoris (Libby, 1995).

1.3.1.6 The necrotic core and plaque rupture

As the plaque progressively gets larger, a fibrous cap is formed, walling off the lesion from the lumen: the fibro fatty atheroma, represents a type of healing response to injury. Growth factors from leukocytes and platelets induce smooth muscle cell proliferation. The lesion is now composed of leukocytes, lipid, and cell debris – contributing to the necrotic nature of the core. The core is a result of apoptosis, necrosis, increased proteolytic activity, and lipid accumulation. These lesions expand at their shoulders by means of continued leukocyte adhesion and migration. The shoulder areas are also the weakest due to foam cell concentration, and a thinning of the cap. There are several factors that influence the risk of plaque rupture, such as; wall stress, location, size, inflammation activity, cap thickness and consistency of the atheromatous core (Naghavi et al., 2003). Shear stress caused by increased blood flow can also influence rupture and consequent thrombosis (Fig. 1.3) (Lee and Libby, 1997).

Fig 1.3: Schematic of the steps leading to the formation of a thrombus (1-4). Initially there is a certain amount of remodelling, preventing a narrowing of the lumen. Eventually the lesion starts to protrude into the lumen, affecting blood flow. (Taken from (Ross, 1999)).

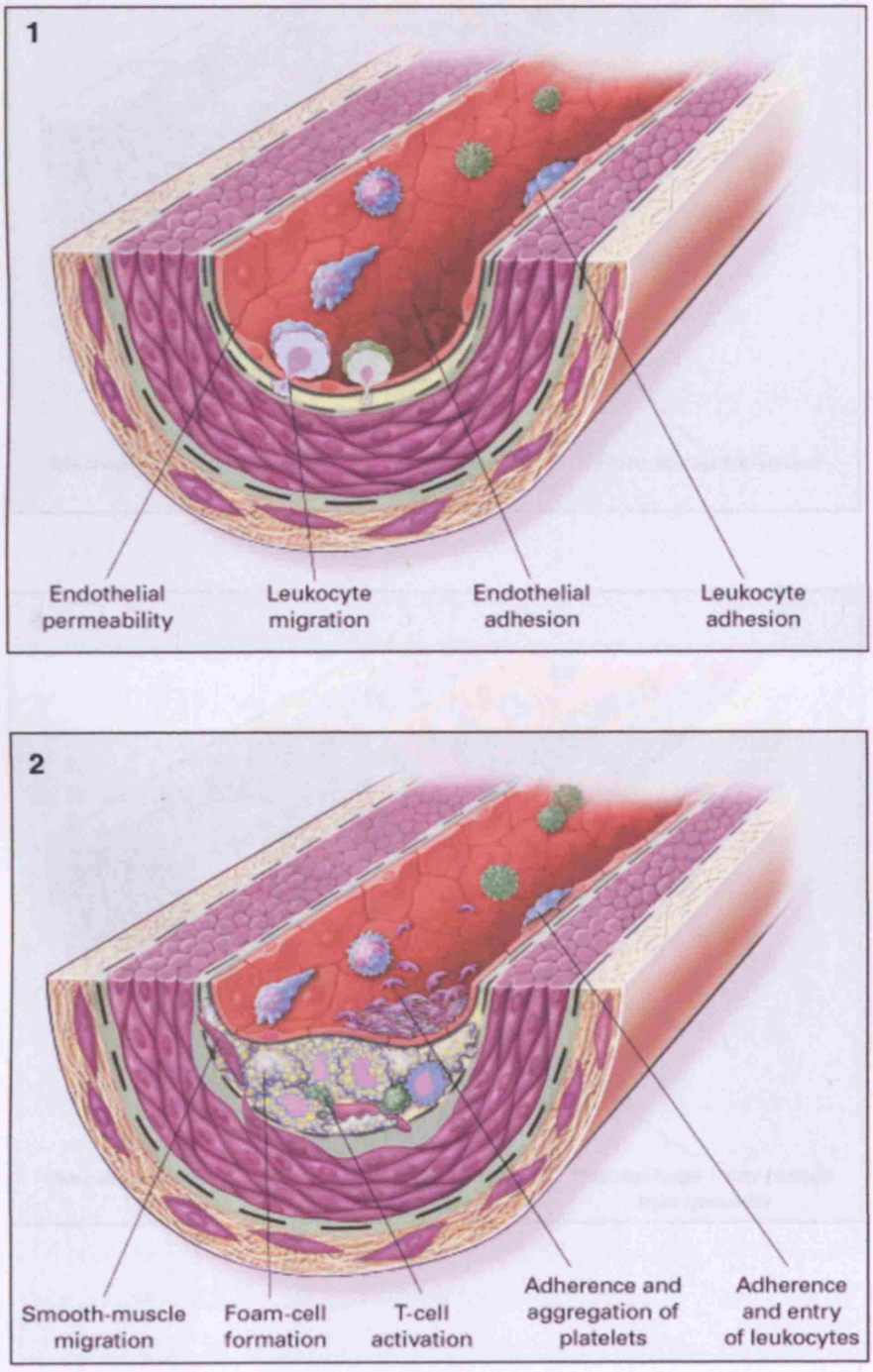
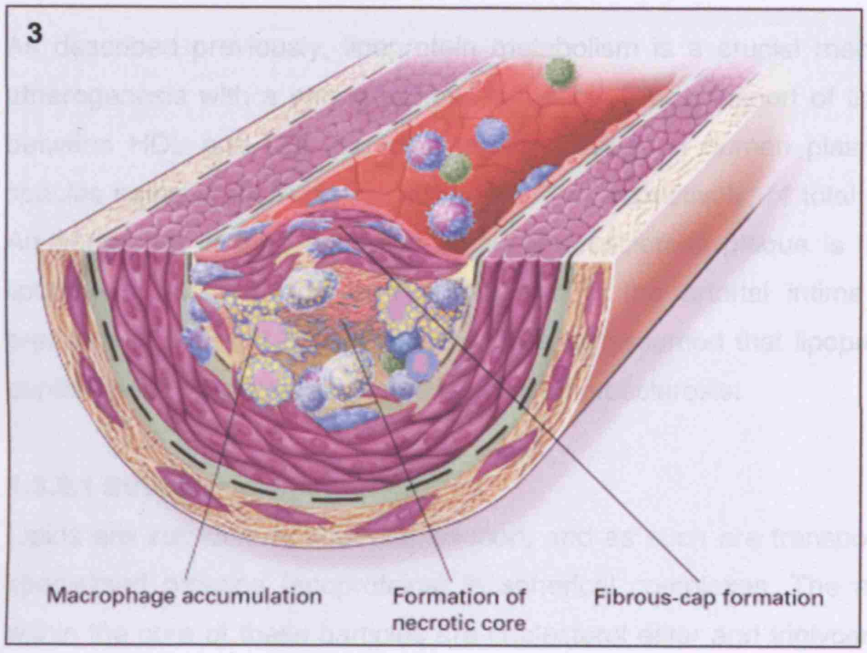
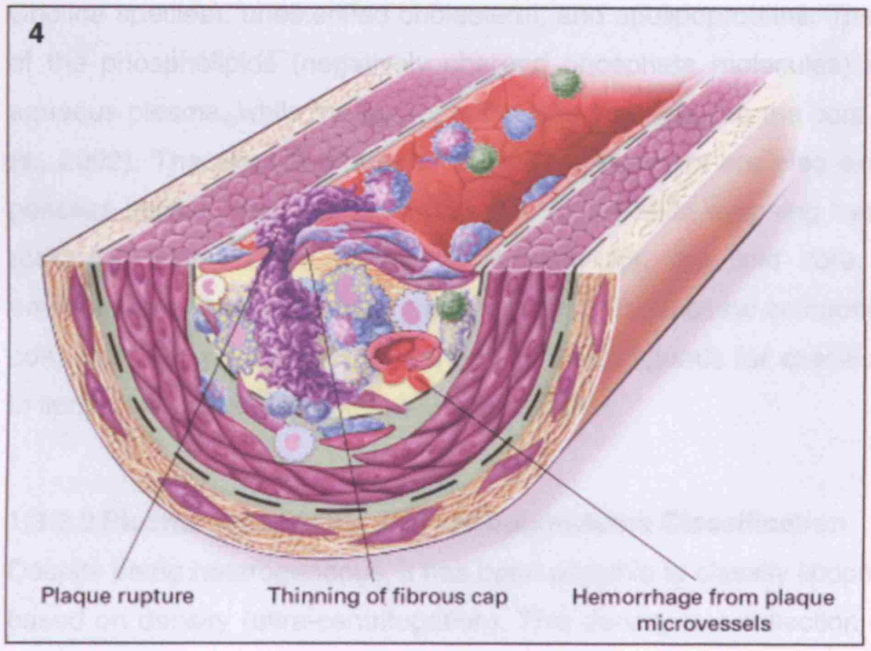


Fig 1.3: continued





1.3.2 Lipoprotein metabolism

As described previously, lipoprotein metabolism is a crucial mechanism involved in atherogenesis with a strong genetic influence. The transport of lipids can be divided between HDL and LDL particles which circulate in human plasma, with these two species being responsible for 20% and 70% respectively, of total plasma cholesterol. An early step in the generation of an atherosclerotic plaque is the accumulation of lipoprotein particles and their aggregates in the arterial intima at sites of lesion predilection (Ross, 1999). It can therefore be assumed that lipoprotein metabolism is central to the initiation and progression of atherosclerosis.

1.3.2.1 Structure of lipoproteins

Lipids are insoluble in aqueous solution, and as such are transported in plasma with specialised proteins (apoproteins) in spherical complexes. The major lipids present within the core of these particles are cholesterol ester and triglycerides (TG). There is an outer layer to the lipoproteins, consisting of Phospholipids (mainly Phosphatidyl Choline species), unesterified cholesterol, and apolipoproteins. The polar head groups of the phospholipids (negatively charged phosphate molecules) are exposed to the aqueous plasma, while the hydrophobic tails protrude into the core (Fig. 1.4)(Horton et al., 2002). The alcohol groups of the free cholesterol are also exposed. Apoproteins possess helical structures, with one side of the helix exposing hydrophilic amino acid residues, enabling the protein to interact with the lipid core, and the aqueous environment. The Apoproteins serve not only as structural components but also act as co-factors for specific enzymes and/or serve as ligands for specific receptors involved in lipoprotein metabolism (Mahley et al., 1984).

1.3.2.2 Plasma Lipoprotein and apolipoproteins Classification

Despite being heterogeneous, it has been possible to classify lipoproteins into 5 groups based on density (ultra-centrifugation). This density is a reflection of the proportion of protein present in these complexes, and these groups can be further subdivided using other techniques that expose electrophoretic mobility, and size of the particles (Table 1.2)(Carmena et al., 2004; Freedman et al., 1998). Another distinguishing feature among the 5 classes are the apoproteins that they associate with. Lipids cannot be synthesised or secreted from the liver and intestine without the corresponding structural apoprotein. These proteins have a diverse range of functions; they can give

structural integrity to lipoproteins, activate enzymes, or act as ligands (Table 1.3)(Feher M.D. and Richmond W., 2000).

Fig 1.4: Schematic of a circulating plasma lipoprotein (Taken from(Horton et al., 2002))

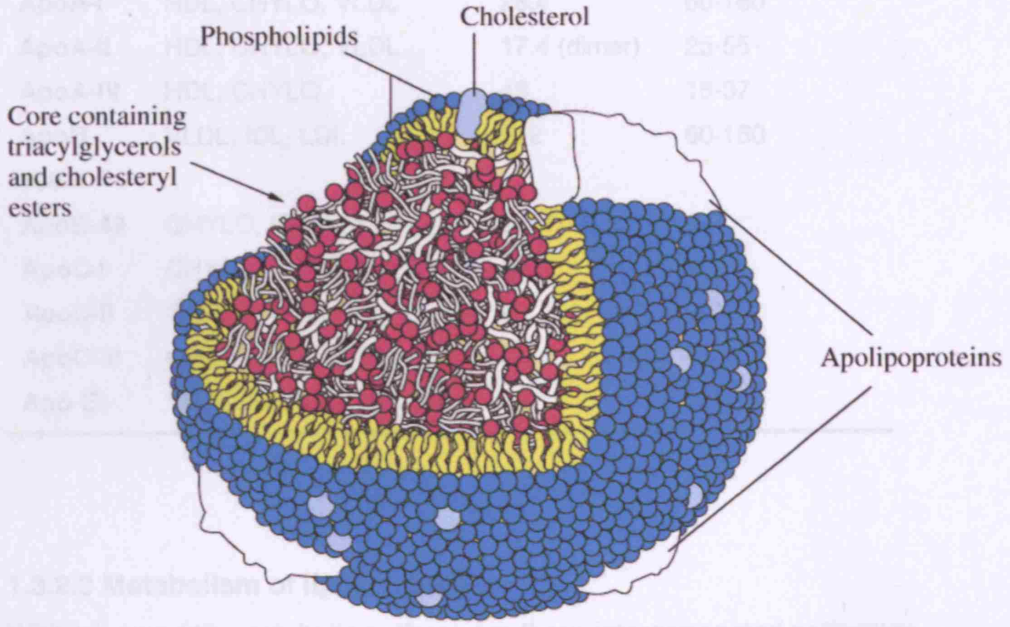


Table 1.2: Broad composition of lipoproteins in human plasma (Taken from (Feher M.D. and Richmond W., 2000))

Lipoprotein		Density (g/ml)	Mean Diameter (nm)	Sources
Chylomicrons CHYLO		<0.95	500	Intestine
Very Low Density Lipoprotein VLDL		<1.006	43	Liver
Intermediate Density Lipoproteins IDL		1.006-1.019	27	Catabolism of VLDL and CHYLO
Low Density Lipoproteins LDL	I	1.02-1.03	27.0	Catabolism of VLDL
	II	1.03-1.04	26.6	
	III	1.04-1.06	26.0	
High Density Lipoprotein HDL	2	1.063-1.125	9.5	Liver + intestine; catab of VLDL and CHYLO
	3	1.125-1.21	6.5	

Table 1.3: Summary of most human Apo-proteins associated with lipoprotein particles (Feher M.D. and Richmond W., 2000).

Apo-protein	Associated Lipoprotein Class	KDa	Plasma Conc. (mg/dL)
ApoA-I	HDL, CHYLO, VLDL	28.4	60-160
ApoA-II	HDL, CHYLO, VLDL	17.4 (dimer)	25-55
ApoA-IV	HDL, CHYLO	46	15-37
ApoB-100	VLDL, IDL, LDL	512	60-160
ApoB-48	CHYLO, IDL(remnant)	245	0-2
ApoC-I	CHYLO, VLDL, HDL	6.6	3-10
ApoC-II	CHYLO, VLDL	9	1-6
ApoC-III	CHYLO, VLDL, HDL	8.8	4-20
Apo-E	VLDL, IDL, CHYLO, HDL	35	2.5-5

1.3.2.3 Metabolism of lipoproteins

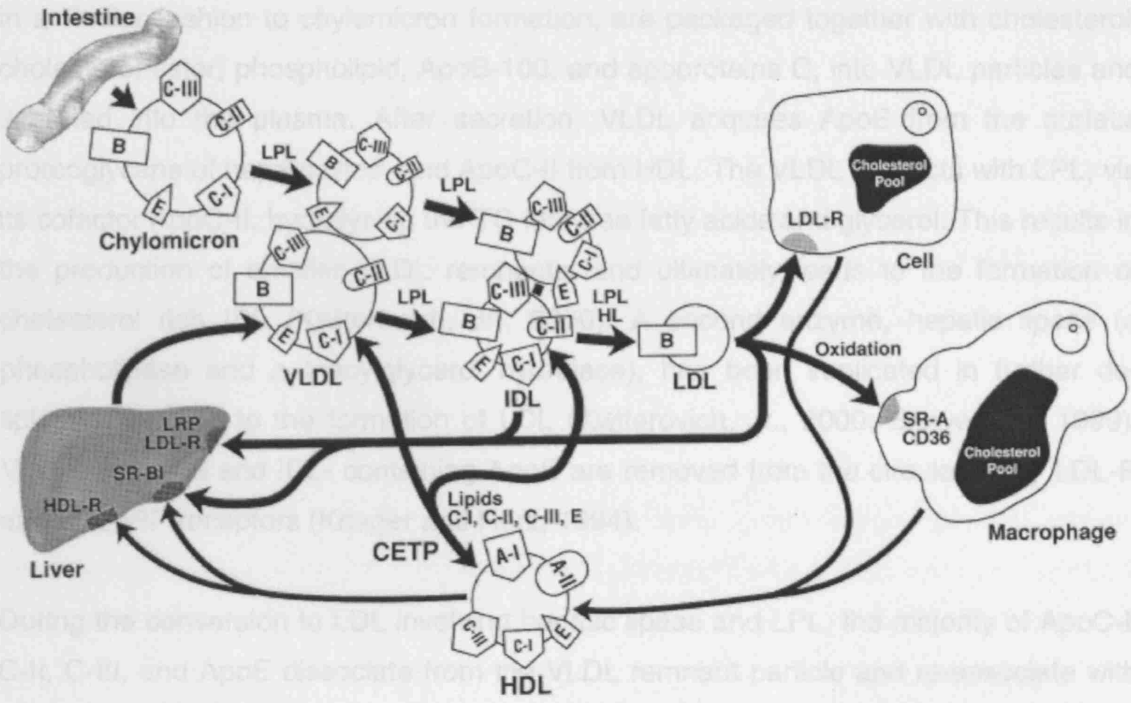
Within lipoprotein metabolism, there are three interconnected pathways:

- The transport of dietary or exogenous fat
- Transport of hepatic or endogenous fat
- Reverse cholesterol transport

These pathways are so interdependent that disruptions in the function of one will affect the products of the other. By understanding these mechanisms, it is therefore possible to give appropriate clinical choices for patients that may be susceptible to CHD (Kwiterovich, Jr., 2000).

Lipoprotein metabolism can be divided into two conceptually different pathways; the ApoB lipoprotein and the HDL pathways (Fig. 1.5). The ApoB pathway consists of a cascade of lipoproteins containing either the ApoB-48 or ApoB-100 isoform secreted from the intestine or liver, respectively. Within this pathway, LDL, VLDL, and lipoprotein (a) are the three major ApoB containing lipoproteins found in blood, and elevated levels of these are associated with CHD (Brewer, Jr., 1999). On the other hand, HDL has protective effects with regards to atherosclerosis through the reverse cholesterol transport system (Zhang et al., 2000; Kwiterovich, Jr., 1998).

Figure 1.5: Overview of lipoprotein metabolism (Taken from (Brewer, Jr., 1999; Kwiterovich, Jr., 2000)).



1.3.2.3.1 Transport of Dietary (exogenous) fat

After intestinal absorption, cholesterol and fatty acids are re-esterified to form triglycerides and cholesterol esters in intestinal mucosal cells. These lipids are packaged with ApoB-48, phospholipid, unesterified cholesterol and several apolipoproteins into nascent chylomicrons, which are then secreted through the thoracic duct, into the plasma (Eisenberg, 1984). The chylomicrons are able to transport dietary lipids to the liver and peripheral tissues. Following this secretion process, the chylomicrons acquire ApoE, ApoC-I, C-II, and C-III from HDL. ApoC-II activates Lipoprotein Lipase (LPL) which hydrolyses the triglyceride in the core, into free fatty acids and glycerol (Eisenberg and Levy, 1975). This remodels the chylomicron into particles with the same density as VLDL and IDL, called 'remnants' (Kwiterovich, Jr., 2000). The remnants are then taken up primarily by liver parenchymal cells, via the LDL receptor related protein (LDL-RP), and the LDL receptor (LDL-R) (Krieger and Herz, 1994; Brown and Goldstein, 1986; Beisiegel et al., 1989). A slowed chylomicron clearance can result in elevated postprandial triglyceride (TG) levels (in control patients TG usually return to normal after 8-10 hours) seen in patients with coronary artery disease (Patsch et al., 1992).

1.3.2.3.2 Transport of Endogenous (hepatic) fat

Fatty acids surplus to oxidative requirements in the liver are esterified to form TG, and in a similar fashion to chylomicron formation, are packaged together with cholesterol, cholesterol ester, phospholipid, ApoB-100, and apoproteins C, into VLDL particles and secreted into the plasma. After secretion, VLDL acquires ApoE from the surface proteoglycans of hepatocytes, and ApoC-II from HDL. The VLDL interacts with LPL, via its cofactor ApoC-II, hydrolysing the TG into free fatty acids and glycerol. This results in the production of smaller VLDL remnants, and ultimately leads to the formation of cholesterol rich IDL (Kwiterovich, Jr., 2000). A second enzyme, hepatic lipase (a phospholipase and a triacylglycerol hydrolase), has been implicated in further de-lipidation leading to the formation of LDL (Kwiterovich, Jr., 2000; Brewer, Jr., 1999). VLDL remnants and IDL- containing ApoE are removed from the circulation by LDL-R and LDL–RP receptors (Krieger and Herz, 1994).

During the conversion to LDL involving hepatic lipase and LPL, the majority of ApoC-I, C-II, C-III, and ApoE dissociate from the VLDL remnant particle and re-associate with HDL (Brewer, Jr., 1999). LDL almost exclusively has ApoB-100 on its surface, and has two possible outcomes: it can firstly be cleared primarily by the receptors present in the liver, but also the adrenal cells, and peripheral cells such as smooth muscle and fibroblasts (Goldstein et al., 1985). However, under certain conditions the LDL may be modified or oxidised, and is then removed by the SR-A or CD36 scavenger receptors present on macrophages (Krieger and Herz, 1994; Steinberg et al., 1989).

1.3.2.3.3 HDL and Reverse Cholesterol transport

To achieve neutral cholesterol balance, the ~6mg/kg body weight of cholesterol that is synthesised by peripheral tissues per day must be efficiently removed (Dietschy et al., 1993). The reverse cholesterol transport system (Fig 1.5) is a method of transferring cholesterol from non-hepatic cells, such as macrophages, to the liver and steroidogenic organs. HDL acquires free cholesterol from non-hepatic tissue which is then esterified by Lecithin- Cholesterol Acyltransferase (LCAT) with Apo A-I as a co-factor. Cholesterol ester is then transferred by CETP from mature HDL particles to ApoB containing lipoproteins (CHYLO/ VLDL), which can then be taken up by the liver by the LDL or LDL-RP receptors (Fielding and Fielding, 1995). HDL also acquires TGs which are hydrolysed by Hepatic Triglyceride Lipase (HTGL) forming smaller particles that bind the Scavenger Receptor type B1 (SR-B1) on the liver or other steroid producing tissues (Assmann and Nofer, 2003).

1.3.3 The generation of an atherogenic lipid profile

As section 1.2.2.3 illustrates, defects in lipid metabolism often result in increased risk of CHD and are seen as one of the more potent initiators of atherosclerosis. An 'atherogenic lipid profile' (or pattern B profile) is generally understood to involve elevated triglyceride levels, low levels of HDL and the predominance of small dense LDL (sdLDL) particles (Packard et al., 2000a). The generation of sdLDL is an important process that has implications in the further modification and oxidation of LDL particles, particularly in the arterial intima of a developing plaque.

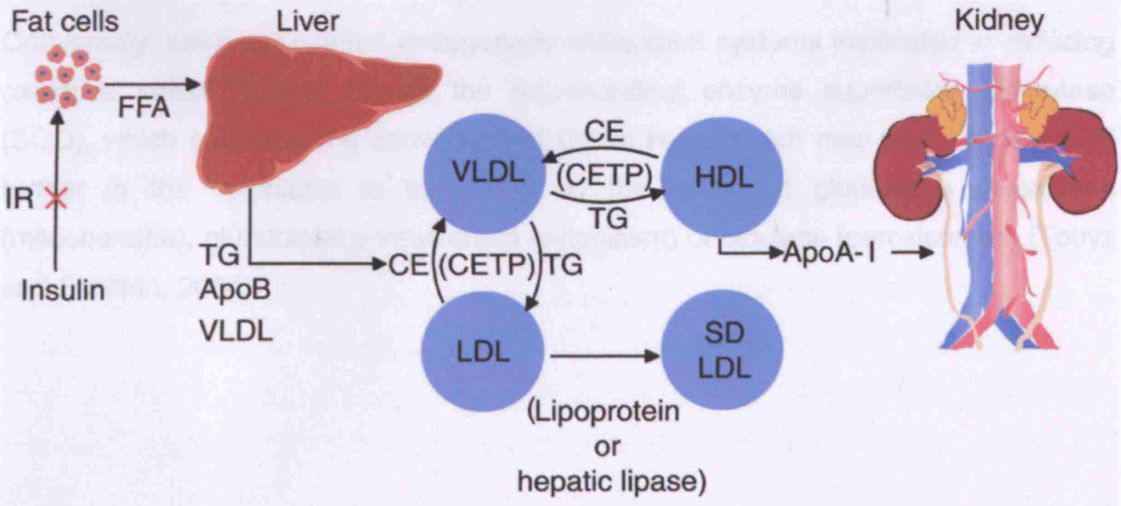
1.3.3.1 Small dense LDL

As table 1.2 illustrates, LDL is itself structurally heterogeneous. Through the use of density centrifugation, it is possible to identify three broad ranges of LDL particle density with LDL-III being the most dense and smallest particle (Packard et al., 2000a). Peak size of LDL particles in human populations exhibits a somewhat bi-modal distribution that can be separated into a buoyant LDL phenotype (pattern A) or a smaller, denser LDL phenotype (pattern B) (Berneis and Rizzo, 2004; Feingold et al., 1992). The small dense LDL phenotype rarely occurs as an isolated disorder, and is commonly accompanied by hypertriglyceridemia, reduced HDL levels, abdominal obesity, insulin resistance (all of which are associated with the metabolic syndrome) and a series of other metabolic alterations affecting endothelial function and thrombosis (Kwiterovich, Jr., 2000; Carmena et al., 2004). A large amount of epidemiological evidence exists to suggest that the generation of sdLDL is firmly associated with CHD risk, in particular those individuals exhibiting insulin resistance. The Québec cardiovascular study found that individuals with elevated levels of plasma insulin and ApoB together with sdLDL particles showed a significant increase in CHD risk (Lamarche et al., 1999). Further analysis in this study population by St. Pierre *et al.* demonstrated that the cholesterol concentration in sdLDL particles showed the strongest association with CHD risk. Multivariate analysis indicated that the relationship between LDL cholesterol levels in particles with a diameter less than 255 angstroms (Å) and CHD risk was independent of all non-lipid risk factors and of LDL, HDL, TGs and Lp(a) levels (St Pierre et al., 2001). Other cross-sectional and prospective studies have identified differences in LDL particle size, density and composition between patients suffering a CHD event and healthy individuals (Austin et al., 1988; Campos et al., 1992; Stampfer et al., 1996). Meanwhile, family studies have indicated that this phenotype is an inherited trait, although it appears that no single gene dominates the phenotype (Packard et al., 2000a).

One proposed metabolic mechanism for the formation of sdLDL involves large visceral fat deposits promoting insulin resistance or a fundamental defect in the incorporation of free fatty acids (FFA) into adipocytes, resulting in an elevation of FFA going to the liver (Fig. 1.6). This can lead to increased TG formation, decreased LDL proteolysis and an increase in VLDL secretion. TG in the core of VLDL exchanges for cholesterol esters in LDL, forming cholesterol depleted LDL particles. Hydrolysis by hepatic lipase of TG in LDL forms a small dense LDL particle. Cholesterol ester on HDL is exchanged via CETP for TG on VLDL and TG enriched HDL is readily catabolised by the kidney, reducing HDL levels and interfering with reverse cholesterol transport (Fig. 1.6)(Ginsberg, 2000).

Small dense LDL has been shown *in vitro* to have an increased capacity to infiltrate into the arterial intima due to its reduced size (Packard et al., 2000a). Once in the intima, sdLDL has a high affinity for proteoglycans and also reduced receptor-mediated uptake, increasing its residence time and potential for further modification (Anber et al., 1996). However, the atherogenicity of sdLDL is greatly enhanced as a result of sdLDL particles susceptibility to oxidation (Tribble et al., 1995). The altered surface lipid layer of sdLDL associated with reduced content of free cholesterol (Tribble et al., 1992) and increased content of polyunsaturated fatty acids might contribute to this enhanced oxidative susceptibility (de Graaf et al., 1991).

Fig 1.6: Insulin Resistance (IR) mechanism of small dense LDL generation (Taken from (Ginsberg, 2000))



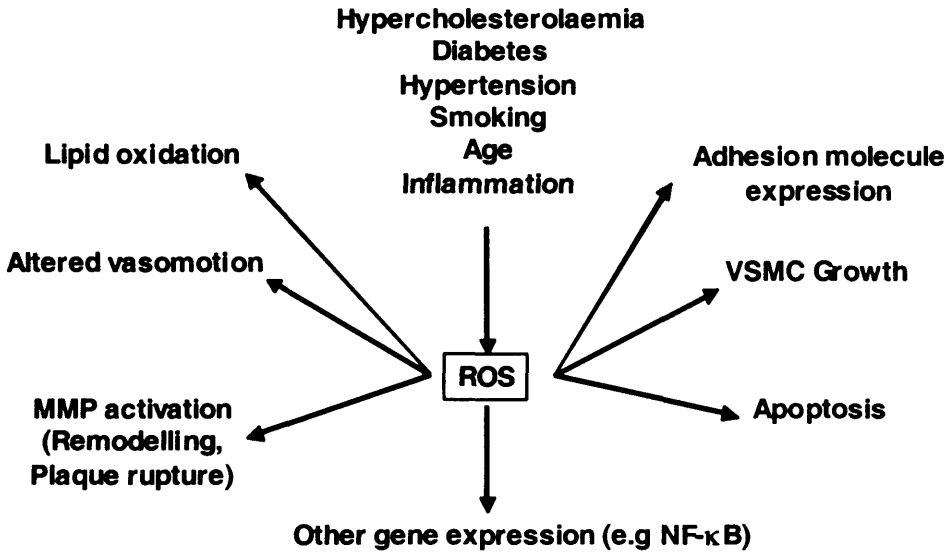
1.3.3.2 Oxidative modification of LDL

1.3.3.2.1 Oxidative stress in vivo

Oxidative stress (OS) results from an imbalance between oxidant production and anti-oxidant defences (Maritim et al., 2003) and is crucial in the modification of susceptible lipoproteins such as sdLDL. The precise mechanisms by which oxidative stress occurs are still unclear. However, one of the more critical aspects of this process *in vivo* is the generation of Reactive Oxygen Species (ROS). ROS are molecules that are ultimately derived from molecular oxygen, but have undergone univalent reduction, so that they readily react with other biological products. ROS include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\cdot}$) and the peroxynitrite radical ($OONO^{\cdot-}$). The oxidative stress generated by free radicals is further enhanced by other processes such as the oxidation of polyunsaturated fatty acids by lipoxygenase enzymes in the cytosol of macrophages (Yla-Herttuala et al., 1990; Yla-Herttuala et al., 1991), and the formation of Advanced Glycation End (AGE) products in hyperglycaemic individuals (another source of superoxide) (Evans et al., 2002). In its most severe form, redox imbalance can result in cell death following widespread macromolecule oxidation, while more subtle changes appear to play a role in modulating a range of signal transduction pathways (Suzuki et al., 1997) and effects related to the progression of atherosclerosis, such as; diabetes, hypercholesterolemia, renal failure, ageing, hypertension and smoking (Fig. 1.7) (Harrison et al., 2003). All molecules are potential targets for ROS (proteins, lipids and DNA), but because of their ubiquitous distribution and their propensity to contain double bonds, unsaturated lipids present in membranes and lipoprotein particles are often targeted (Evans et al., 2002).

Conversely, there are several endogenous antioxidant systems implicated in reducing oxidative stress. These include the mitochondrial enzyme superoxide dismutase (SOD), which catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2, which may then be detoxified further in the cytoplasm to form H_2O by the action of glutathione peroxidase (mitochondria), glutathione s-transferase (cytoplasm) or catalase (peroxisomes) (Touyz and Schiffrin, 2004).

Fig 1.7: The potential causes and consequences of ROS imbalance (courtesy of Dr Jeff Stephens)



ROS: Reactive oxygen species, MMP: Matrix metalloproteinases, VSCM: Vascular smooth muscle cell

1.3.3.2.2 Oxidised LDL and its association with atherosclerosis

It is clear that elevated levels of LDL are recognised as being important in the progression of atherosclerosis (Berliner et al., 1997; Berliner and Heinecke, 1996). Studies have also identified immuno-histochemically, the presence of lipoprotein-like particles with oxidative damage, and lipid oxidation products within lesions (Berliner and Heinecke, 1996). Clinically, elevated levels of oxLDL have been found to be independently associated with increased atherosclerotic burden and CHD risk (Toshima et al., 2000; Weinbrenner et al., 2003). People with T2DM appear to be particularly at risk from oxidative modification of LDL due to these individuals being associated with an increase in oxidative stress (Cai and Harrison, 2000; Davi et al., 1999). A recent study has demonstrated that diabetic individuals show higher levels of oxLDL compared to control subjects (Phillips et al., 2005). However, it is still unclear as to whether diabetes is the cause or a consequence of oxidative stress. A possible link could be that hyperglycaemia may result in the enzymic glycosylation of proteins, eventually resulting in AGE products and enhanced ROS production (Evans et al., 2002).

Oxidised-LDL *in vivo* and *in vitro* has been found to be associated with atherosclerotic processes within the vessel wall such as the chemotaxis of monocytes, monocyte adhesion to endothelial cells, foam cell formation, up-regulation of apoptotic pathways, migration and proliferation of SMC, and pro-coagulant properties (Quinn et al., 1988; Chisolm and Steinberg, 2000) (Table 1.4). Whether the initial step of oxidative modification of LDL occurs in the plasma or in the elevated oxidative stress environment of the arterial intima is still the centre of debate, with a recent study showing that *plasma* levels of oxLDL positively correlated with those individuals suffering from CHD (Nordin et al., 2003). However, within the vessel wall there are several enzyme sources of oxidants, such as NAD(P)H oxidases (enzymes that utilise NADH and NADPH in order to generate superoxide anions), nitric oxide synthases (generating nitric oxide or peroxynitrate radicals), myeloperoxidase (a source of non-radical hydrogen peroxide), and members of the lipoxygenase enzyme family [reviewed in (Stocker and Keaney, Jr., 2005)]. The oxidative environment within the intimal space caused by these enzymes can be exacerbated by the properties of sdLDL.

In addition, many of the biological effects of oxLDL are poorly defined since oxLDL contains a complex, variable, incompletely characterised mixture of oxidation products (Carpenter et al., 2003). However, some of the effects of oxLDL (and minimally modified-LDL) are related to the generation of inflammatory mediators, namely, eicosanoids (a variety of compounds encompassing prostaglandins, thromboxanes, hydroxyl/epoxy- fatty acids, lipoxins and isoprostanes) from phospholipid precursors such as arachidonic acid (AA) and similar polyunsaturated fatty acids present in the lipid bi-layer of LDL (De Caterina and Zampolli, 2004). Members of the lipoxygenase and PLA2 enzyme family are able to catalyse the generation of AA and its consequent conversion into leukotrienes. PLA2 enzymes bound to LDL particles are able to hydrolyse oxidised phospholipids and can generate significant concentrations of the free fatty acid, AA (Balsinde et al., 2002; Oestvang et al., 2004). 5-Lipoxygenase (5-LO) initiates the leukotriene biosynthetic pathway by the conversion of AA into leukotriene A_4. Further hydrolysis leads to the production of leukotriene B_4, itself a chemo attractant for neutrophils and macrophages, as well as inducing the adhesion of these cells to the vascular endothelium. Metabolites of leukotriene B_4 have also been implicated in vascular vaso-constriction (De Caterina and Zampolli, 2004).

Table 1.4: Consequences of elevated oxLDL (Courtesy of Dr Jeffrey Stephens)

Increased foam cell formation
Increased monocytes & T-cells chemotaxis
Increased vascular smooth muscle and macrophage production
Altered gene expression (MCP-1, IL-1, ICAMs)
The induction of pro-inflammatory genes
(PPARγ, Haemoxygenase, SAA, Caeruloplasmin)
Increased Immunogenicity (elicits autoantibody formation & activated T-cells)
Increased LDL susceptibility to aggregation
Enhanced pro-coagulant pathways
Altered arterial vasomotor properties

1.3.3.2.3 Anti-oxidative effects of HDL

There are several endogenous systems involved in the breakdown of lipid peroxidation products, one of which is HDL. Until relatively recently, HDL was thought of as exerting an anti-atherogenic effect through the reverse cholesterol transport system. However, HDL's ability to acquire oxidation products from LDL, and the presence of anti-oxidants such as ApoA-I are also thought to be important (Mackness and Durrington, 1995). Indeed, it has been shown that HDL totally inhibits mild oxidative modification of LDL in co-cultures of human aortic wall cells (Navab et al., 1991). However, it has now become evident that there are anti-oxidant mechanisms in HDL that may in some part be due to enzymic activity. Several enzymes associated with HDL have potential anti-atherogenic properties; Paraoxonase (PON) (Mackness et al., 1991; Mackness et al., 1993), Platelet Activating Factor Acetylhydrolase (PAFAH or Lp-PLA2) (Marathe et al., 2003; Noto et al., 2003; Watson et al., 1995), and Glutathione peroxidase (GPX) prevent the formation / degradation of products from lipid peroxidation (Chen et al., 2000; Sattler et al., 1994) and could themselves be involved in arresting the progression of atherosclerosis.

1.4 The Phospholipase A2 family

1.4.1 Classification

The phospholipase A2 (PLA2) group of enzymes are diverse in their structure, location on the chromosome, and secondary function. However, they do share some core properties that enable them to be classified together. The essential criterion for PLA2 activity is the ability to catalyse the *sn-2* ester bond of a phospholipid substrate, generating free fatty acids and lyso-phospholipids (Fig. 1.10) (Six and Dennis, 2000). Naturally occurring substrates include Platelet activating factor (PAF), short and long fatty acid chain phospholipids. Certain PLA2 enzymes have preferences for different lengths of *sn-2* acyl chains, for instance; Platelet Activating Factor Acetyl-Hydrolase (PAFAH or Lp-PLA2) has a marked preference for acyl chains up to nine methylene groups, and is inhibited strongly by longer chains (Stremler et al., 1989). In general, most PLA2 enzymes are able to hydrolyse chains at the sn-2 position between two (acetyl) and 20 (arachidonate) carbons in length (Six and Dennis, 2000).Because this class of enzymes have varied secondary functions, they do not always exhibit similar categorisation, and are listed under several different names. A summary of some of the more common groups of phospholipase A2 enzymes is listed in table 1.5 (taken from (Jaross et al., 2002)).

1.4.1.1 Phospholipase A2 enzymes utilising a catalytic histidine

Groups I, II, V, and X PLA2 enzymes share a common mechanism for cleavage of the *sn-2* ester bond. Hydrolysis is achieved by the activation and orientation of a water molecule by hydrogen binding to the active site histidine, which dictates the pH dependence of 7-9 of all PLA2 enzymes with a catalytic histidine. Located adjacent to the histidine is a conserved aspartate which binds calcium ions (hence the millimolar dependency), important in the stabilisation of the transition site of the PLA2 reaction (Dennis, 1994). These Phospholipase A2 enzyme groups are closely related in terms of structural homology, with conserved disulphide bonds and a cleaved signal peptide. One of the first enzymes characterised within this family is a group IIA PLA2, and has been mapped to chromosome position 1p34-36, clustered with another four PLA2 genes, of which group V is the closest (Tischfield et al., 1996).

1.4.1.2 Group VII Phospholipase A2 family

Group VII PLA2 enzymes are distinct from PLA2 enzymes utilising a catalytic histidine. Site directed mutagenesis has identified a classic Hydrolase triad of Ser-273, Asp-296

and His-351 in these enzymes, which is conserved across a range of vertebrates (Tjoelker et al., 1995a). Group VII A and B enzymes are α/β hydrolases with catalytic triads lacking a conserved aspartate that binds to calcium, therefore making them Calcium independent (Derewenda and Ho, 1999).

Table 1.5: Summary of validated PLA2 enzymes (Taken from (Jaross et al., 2002)).

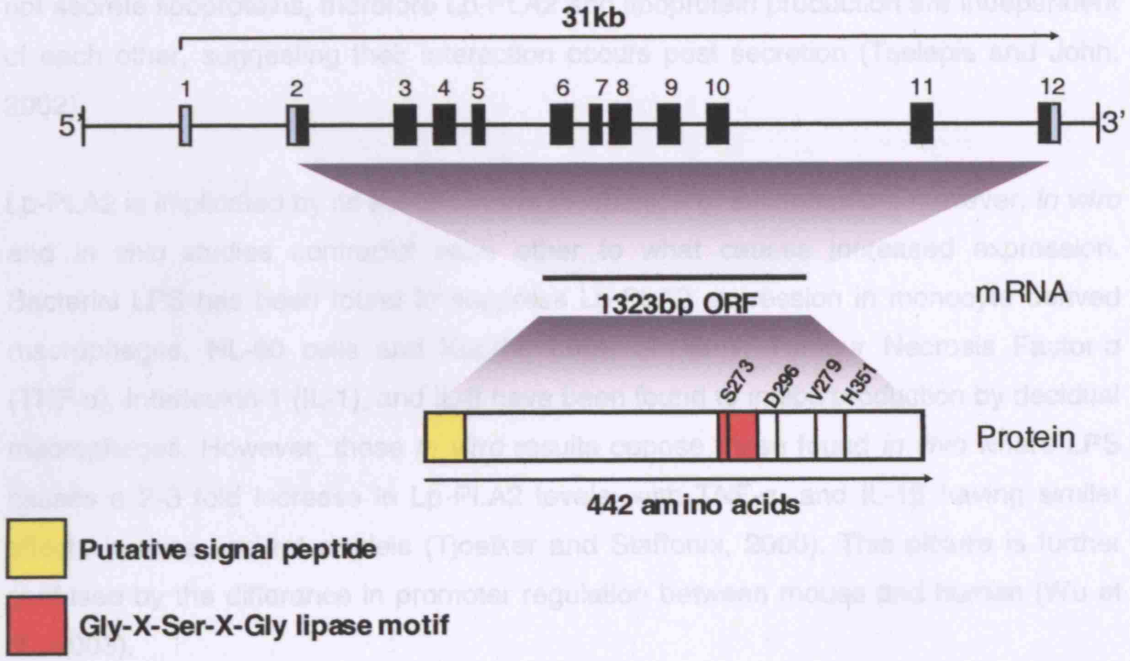
Group		Common source	Alternate name	Location	Size (kDa)	Disulfides	Ca^{2+} requirement	Human chromosome
I	A	snake venoms (cobras, kraits)		secreted	13–15	7	mM	N/A
	B	porcine/human pancreas		secreted	13–15	7	Mm	12q23–24
II	A	snake venoms (rattlesnakes, vipers), human synovia, platelets		secreted	13–15	7	mM	1p34–36
	B	Gaboon viper		secreted	13–15	6	mM	
	C	rat/mouse testes		secreted	15	8	mM	1p34–36
III	D, E, F	mouse, human cells		secreted	14–16	7		
III		bee, lizard, scorpion, human		secreted	16–18	5	mM	22q
IV	A	human U937 cells/platelets, rat kidney	$cPLA_2\alpha$	cytosolic	85	–	μM	1q25
	B	human pancreas liver/heart/brain	$cPLA_2\beta$	cytosolic	100	–	μM	15
	C	human heart/skeletal muscle	$cPLA_2\gamma$	cytosolic	65	–	none	19
V		mammal heart, lung, mast cells, macrophages		secreted	14	6	mM	1p34–36
VI	A–1	$P338D_1$ macrophages, CHO cells	$iPLA_2$	cytosolic	84–85	–	none	22q13.1
	A–2	human B-lymphocytes, testis	$iPLA_2$	cytosolic	88–90	–	none	22q13.1
	B	human heart/skeletal muscle	$iPLA_2$	cytosolic	88	–	none	7q31
VII	A	human plasma	PAFAH, Lp-PLA2	secreted	45	–	none	ND
	B	bovine brain	PAFAH	cytosolic	40	–	none	ND
VIII	A	human brain		cytosolic	26	–	none	ND
	B	human brain		cytosolic	26	–	none	11q23

1.4.2 Platelet Activating Factor Acetylhydrolase (PAFAH), Lipoprotein-associated Phospholipase A2 (Lp-PLA2), *PLA2G7*

1.4.2.1 General Properties

There are two forms of functioning PAFAH identified in the human body, intracellular (also referred to as cytosolic) and extracellular (plasma or secretory) pools (Six and Dennis, 2000). Several differing plasma PAFAH isoforms have been purified and cloned, with seemingly totally different structures and specificities. For instance, PAFAH I and II (Hattori et al., 1996; Ho et al., 1997) are plasma enzymes involved in cell apoptosis and nervous system formation (Arai et al., 2002; Stafforini et al., 1997). However, the most common extracellular form of plasma PAFAH (termed Lp-PLA2 due to its association with lipoproteins) was originally purified in 1987 (Stafforini et al., 1987b). The Lp-PLA2 gene (*PLA2G7*) contains 12 exons and maps to the short arm of chromosome 6 p12-21.1 (Fig. 1.8) (Tew et al., 1996). The protein is made up of 442 amino acids and contains a lipase consensus motif of Gly-X-Ser273-X-Gly. In addition, site directed mutagenesis has identified a highly conserved hydrolase triad of Ser-273, Asp-296 and His-351 (Tjoelker et al., 1995a). Lp-PLA2 is regarded as a α/β hydrolase, yet lacks a conserved aspartate that binds to calcium, therefore making the enzyme calcium independent unlike many other PLA2 family members (Derewenda and Ho, 1999). The mature protein has 7 disulphide bridges and is resistant to proteolysis by reagents targeting sulphydryl or histidyl residues (this confers stability in plasma). Lp-PLA2 in plasma is N-glycosylated and contains about 9KDa of a heterogeneous asparagines-conjugated sugar chain(s) involving sialic acid (Tew et al., 1996; Tselepis et al., 2001; Tselepis and John, 2002). To date, it has proved difficult to elucidate the precise quaternary structure of Lp-PLA2 due to the enzymes lipophilic properties.

Fig 1.8: Schematic of the PAFAH (Lp-PLA2) gene located on chromosome 6. Black boxes represent coding exons. The putative peptide signal represents the first 17 amino acids, and the Gly-X-Ser-X-Gly motif covers 4 amino acids. S273, D296 and H351 form part of the catalytic triad, and the V279 is essential for activity.



1.4.2.2 Expression of Lp-PLA2

Lp-PLA2 is secreted into the plasma by platelets, neutrophils, macrophages (expression levels elevated upon monocyte differentiation (Elstad et al., 1989)), T-lymphocytes (Tjoelker and Stafforini, 2000), and tissue sources that are consistent with the haematopoietic origin of the enzyme, such as lymph nodes, the thyroid gland (Cao et al., 1998; Tjoelker et al., 1995b), and liver (Howard et al., 1997). Indeed, when patients received an allogenic bone marrow transplant from a donor with a known mutation that completely knocks out Lp-PLA2 secretion (V279F), their Lp-PLA2 activity was knocked out as well, suggesting that most of the serum Lp-PLA2 activity found in humans is of a haematopoietic origin (Asano et al., 1999).

Approximately 70-80% of the plasma enzymatic activity of Lp-PLA2 is associated with LDL particles, with the remainder residing on HDL (Guerra et al., 1997; Stafforini et al., 1987a; Tjoelker and Stafforini, 2000). Among LDL subspecies, the enzyme is thought to bind preferentially to the more dense LDL sub fractions (Eisaf and Tselepis, 2003). Three amino acid residues; Tyr-205, Leu-116, Trp-115 are critical for the binding of plasma Lp-PLA2 to LDL particles and may involve an interaction with Apo-B (Stafforini

et al., 1999). The relative distribution of Lp-PLA2 between LDL and HDL can also be influenced by the presence of high levels of Lp(a). Some studies have found evidence that Lp(a) contains several-fold greater Lp-PLA2 activity compared with LDL (Blencowe et al., 1995; Eisaf and Tselepis, 2003; Karabina et al., 1996). Haematopoietic cells do not secrete lipoproteins, therefore Lp-PLA2 and lipoprotein production are independent of each other, suggesting their interaction occurs post secretion (Tselepis and John, 2002).

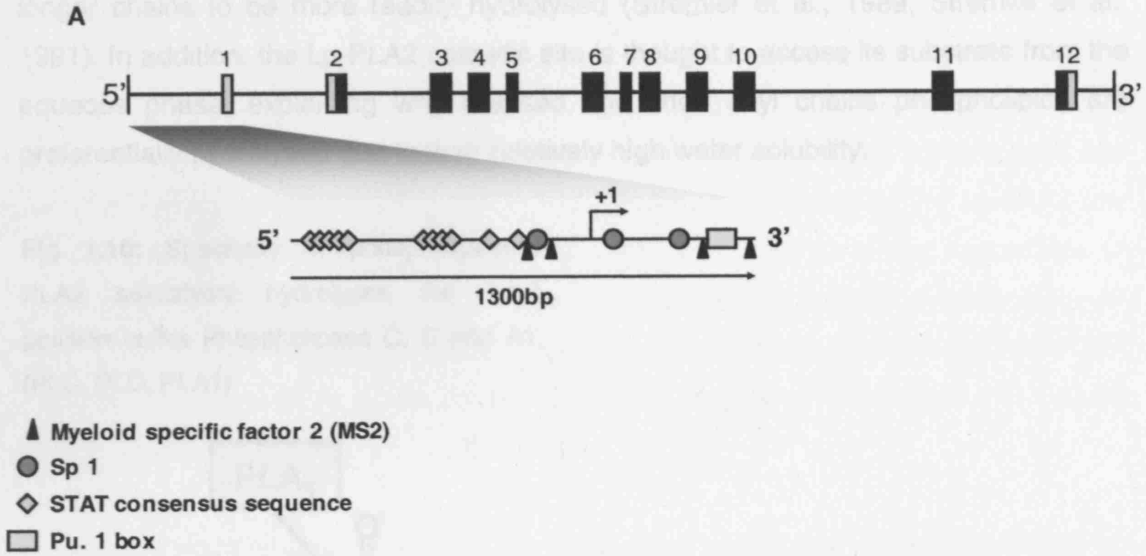
Lp-PLA2 is implicated by its actions in the modulation of inflammation; however, *in vitro* and *in vivo* studies contradict each other to what causes increased expression. Bacterial LPS has been found to suppress Lp-PLA2 expression in monocyte derived macrophages, HL-60 cells and Kupffer cells. Similarly, Tumour Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), and IL-8 have been found to inhibit production by decidual macrophages. However, these *in vitro* results oppose those found *in vivo* where LPS causes a 2-3 fold increase in Lp-PLA2 levels, with TNF-α, and IL-1β having similar effects in mice and rat models (Tjoelker and Stafforini, 2000). This picture is further confused by the difference in promoter regulation between mouse and human (Wu et al., 2003).

1.4.2.3 Transcriptional control and expression of Lp-PLA2 (*PLA2G7*)

The difference in expression levels of Lp-PLA2 when monocytes differentiate is probably the result of promoter regulation. Promoter activity in macrophagic cell lines (RAW264.7 and P388D1) is greater than that in a monocytic cell line (U937) (Cao et al., 1998). Sequencing 1kb 5' (300bp 3') of the transcription initiation start site, reveals numerous cis-acting promoter elements (Fig 1.9, A): Sp1 sites, MS2 binding consensus sequences, one Pu.1 box, and eleven STAT binding consensus sequences (Cao et al., 1998). The transcription factor MS1 is exclusively expressed in monocytic cells, and the Pu.1 box is only expressed in macrophages and B cells: the presence of these two factors in the Lp-PLA2 promoter suggests that the expression of the gene is myeloid specific. The MS2 sites are expressed only by differentiated myeloid cells, suggesting that Lp-PLA2 is indeed under strict differentiation control. The STAT consensus sequences may also influence a response with Interferon-γ (IFN-γ) through the JAK-STAT pathway. The precise signalling pathways invoked by PAF and LPS are not clearly defined. Cao *et al.* (Cao et al., 1998) prepared 5' genomic fragment constructs of varying size fused to a luciferase gene, and transfected COS-7 cells. The results showed that a short 72bp 5'-flanking region was sufficient for 65% of basal activity, and

that there was more than one area in the 1.3kb 5' genomic sequence conferring promoter activity (Fig 1.9, B).

Fig 1.9: Schematic of the proximal promoter of Lp-PLA2 (A) and luciferase reporter assay constructs (B) (Taken from (Cao et al., 1998)).



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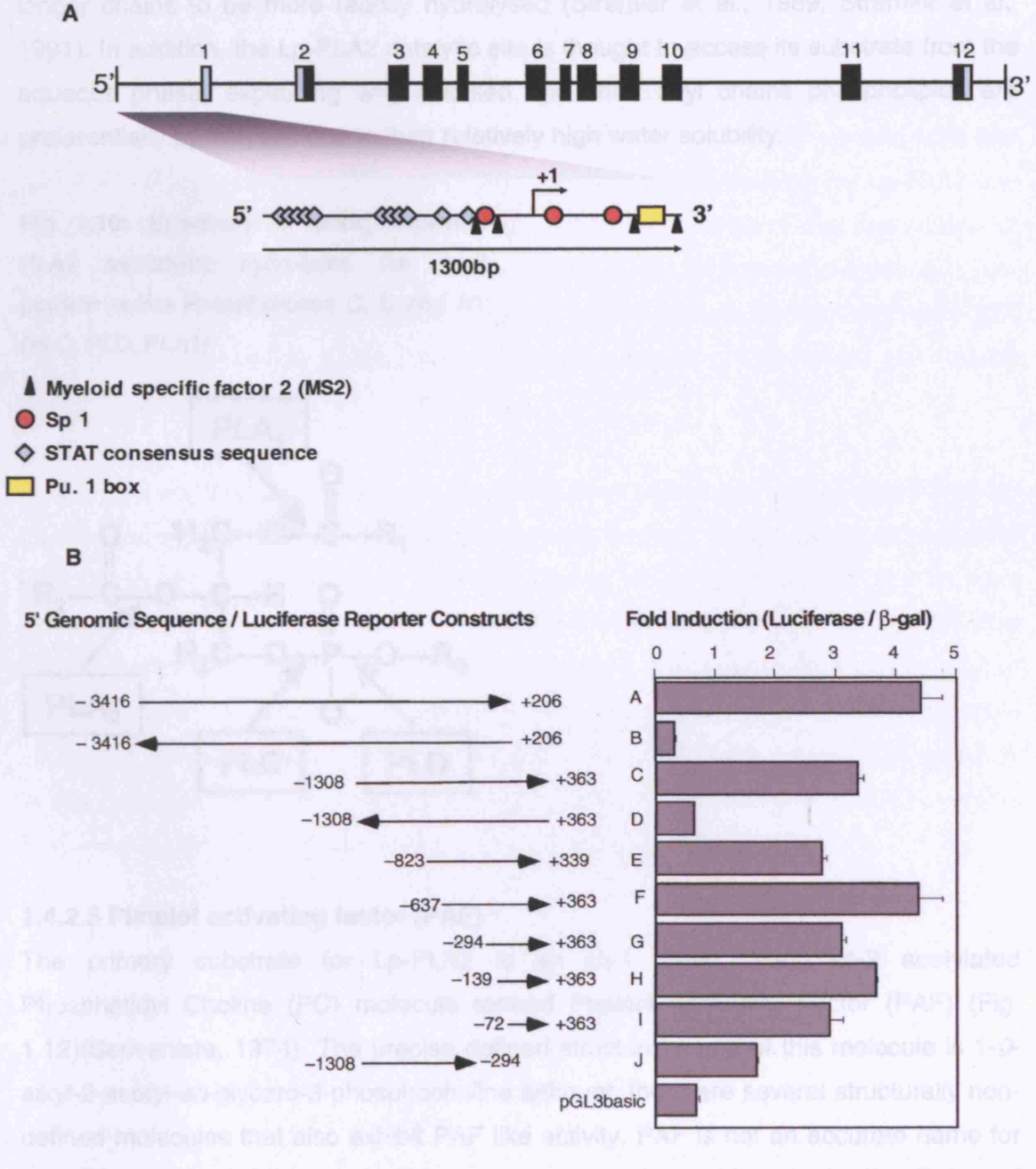
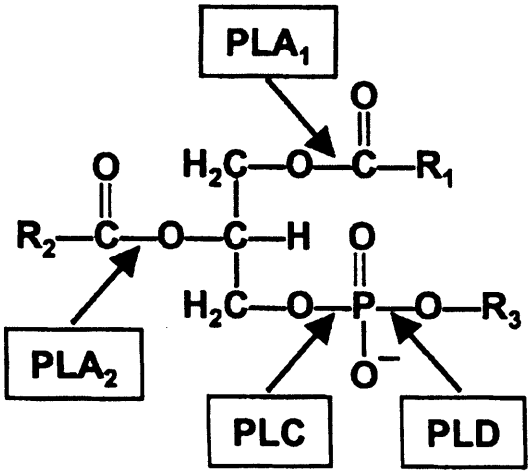


1.4.2.4 Lp-PLA2 substrate specificity

Phospholipase A2 enzymes only cleave glycerophospholipids at the *sn-2* position (Fig. 1.10). However, Lp-PLA2 appears to have a substrate preference for short acyl chains at the *sn-2* position with less than 9 carbon acyl chains (Phosphatidylcholine), unless there is the presence of an aldehyde group (often a product of oxidation) enabling longer chains to be more readily hydrolysed (Stremler et al., 1989; Stremler et al., 1991). In addition, the Lp-PLA2 catalytic site is thought to access its substrate from the aqueous phase, explaining why oxidised and short acyl chains phospholipids are preferentially hydrolysed due to their relatively high water solubility.

Fig 1.10: Specificity of phospholipases. PLA2 selectively hydrolyses the *sn-2* position unlike Phospholipase C, D and A1 (PLC, PLD, PLA1)



1.4.2.5 Platelet activating factor (PAF)

The primary substrate for Lp-PLA2 is an sn-1 ether linked, sn-2 acetylated Phosphatidyl Choline (PC) molecule termed Platelet Activating Factor (PAF) (Fig. 1.12)(Benveniste, 1974). The precise defined structural name of this molecule is 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine although there are several structurally non-defined molecules that also exhibit PAF like activity. PAF is not an accurate name for the diverse functions these molecules are involved in; although the mediation of inflammation and its possible role in diseases, encompassing cardiovascular, cerebral, respiratory, gastro-intestinal, reproductive, dermal, renal, hepatic, and pancreatic systems, are thought to be the most important (Tjoelker and Stafforini, 2000). There are several cell types known to produce and secrete PAF, including basophils,

endothelial cells, neutrophils, eosinophils, macrophages, monocytes, mast cells, and spermatozoa. PAF is also able to activate these cells at very low concentrations ($10^{-10/-12}$M) via its interaction with a cell surface G-protein-coupled receptor, leading to physiological inflammation (Izumi and Shimizu, 1995). Mouse studies using a PAF receptor knock-out have shown a marked broncho-constriction in response to intra-venously administered PAF (Ishii et al., 1997). As a consequence of its potent physiological properties, PAF is tightly regulated. The regulation of this molecule is balanced between a *de novo* synthesis/remodelling pathway involving the combined action of cytosolic phospholipase A2/acyl-coenzyme A-independent transacylase and lyso-PAF: acetyltransferase (Fig. 1.11) (Ninio, 2005); and degradation by Lp-PLA2 into acetate and Lyso-PAF (Snyder, 1995) (Fig. 1.12). The products of this degradation of modified phosphatidylcholine (PC) and PAF, namely Lyso-phosphatidylcholine (Lyso-PC) and Lyso-PAF are endowed with biological activity through an interaction with G2A receptors (Kabarowski et al., 2001), but at relatively higher concentrations (10-100μM) compared to PAF (Kume et al., 1992).

A heterogeneous group of molecules have also been associated with eliciting the same effects observed by PAF (these molecules actually account for a large proportion of the effects thought to be mediated by PAF). These bioactive lipids are derived from oxidative modification of the *sn*-2 arachidonyl moiety of cell membrane and plasma phosphatidylcholines, and can be recognised by the PAF receptor (Smiley et al., 1991). PAF-like molecules are also substrates for Lp-PLA2. However, PAF-like molecule production is not regulated, with their plasma concentration significantly increased by smoking, inflammation, and reperfusion injury. Lp-PLA2 is essentially the only enzyme that is able to determine these levels *in vivo* (Tjoelker and Stafforini, 2000).

Fig 1.11: Pathways of de novo and remodelling of PAF. Oxidised phospholipid PAF-like pathways also shown in the bottom right of the figure with their putative role in the activation of the PAF receptor. Lyso-PC/PAF signalling through the G2A receptor is also shown in the bottom left (Taken from (Ninio, 2005)).

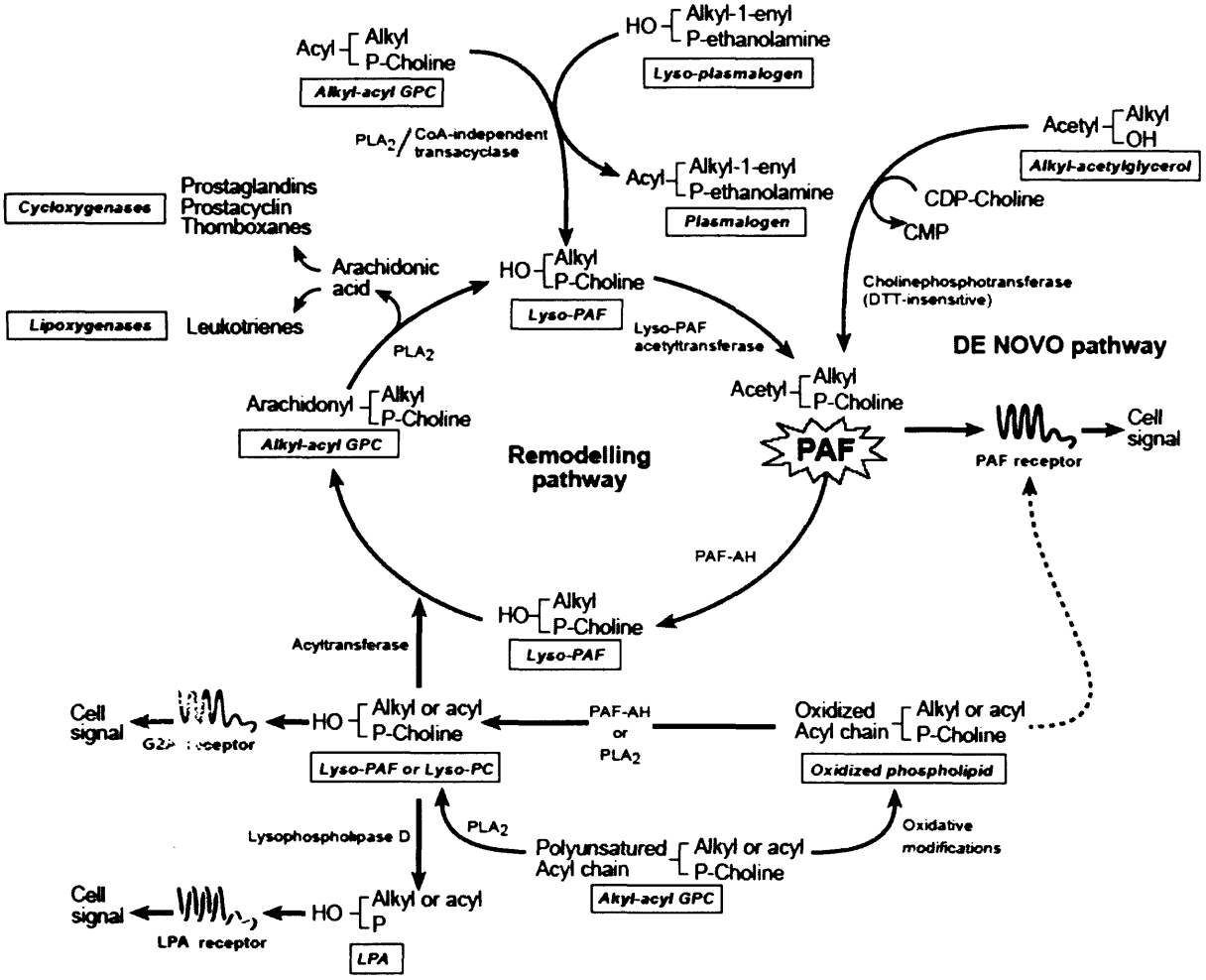
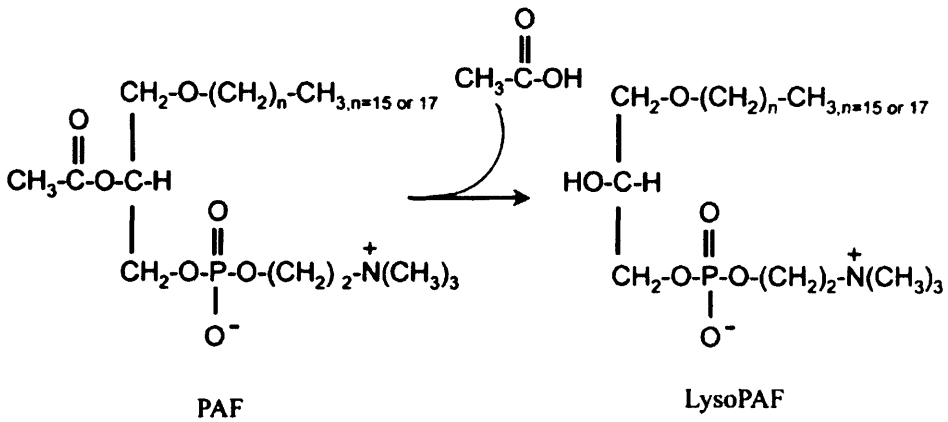


Fig 1.12: Lp-PLA2 catalysed reaction of PAF generating lyso-PAF and acetate (Taken from (Karasawa et al., 2003)).



1.4.2.6 Genetic variation in the *PLA2G7* gene

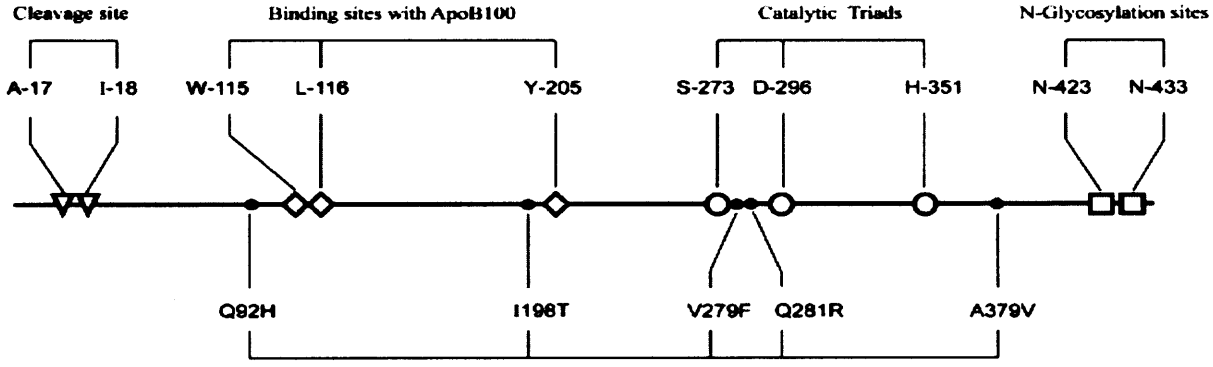
Lp-PLA2 levels are known to vary as much as five fold within a normal healthy population. When plasma activity was measured in 240 individuals from 60 nuclear families (two parents and two eldest offspring), 60% of the variation in activity was accounted for by genetic factors (Guerra et al., 1997).

In 1998, Miwa *et al.* (Miwa et al., 1988) reported that 32 of 816 healthy Japanese adults, and 8 out of 211 children, had negligible Lp-PLA2 activity. The LDL levels between the groups were similar, and the probability of having Lp-PLA2 deficiency was found to be significantly higher in asthmatic children. Cloning and expression studies subsequently identified the causative point mutation in exon 9 that leads to complete abolishment of Lp-PLA2 activity (Tew et al., 1996). Analysis showed that the nucleotide change was G994T. This Single Nucleotide Polymorphism (SNP) leads to a change from Valine 279 in the mature protein, to Phenyl-alanine (Stafforini et al., 1996). Valine at this position is highly conserved across bovine, dog, mouse, and chicken species; and is within 6 amino acids of the important active-site serine (Fig. 1.13)(Tjoelker et al., 1995a). Studies concluded that this polymorphism is responsible for the majority of Lp-PLA2 deficiencies seen in Japan (Stafforini et al., 1996). However, of 80 people lacking Lp-PLA2 activity, 8 did not have this change. Subsequent sequencing of exon 9 in these individuals revealed a previously unidentified A1001G missense mutation, resulting in a Gln281Arg substitution, in a 72-year-old woman with CHD, essential hypertension, and no plasma activity (Yamada and Yokota, 1997). Again, this mutation lies close to the active site serine (Fig. 1.13).

The two mutations listed above have not been found in Caucasian populations, making it hard to study the effects of these polymorphisms on disease states. However, several other SNPs have been identified that have a direct effect on the efficiency by which PAF and other phospholipids are hydrolysed. Kruse *et al.* (Kruse et al., 2000) used Single Strand Conformation Polymorphism (SSCP) analysis and subsequent sequencing to identify three common variants in exons 4, 7 and 11. The exon 4 change was a G275GA, resulting in an alteration of the protein sequence Arg92His. The rare allele frequency of 92His varied between 23% and 25% in atopic and asthmatic individuals from Germany and the UK. The Exon 7 change was a T593C resulting in a Ile198Thr change. The rare allele frequency for this polymorphism in the same cohorts varied from 0.8% to 11.7%. The exon 11 change was a T1136C SNP producing an Ala379Val change, with a rare allele frequency between 10% and 22% (Fig. 1.13). *In vitro* studies suggested that the Ala379Val change led to a two fold increase in the Km

(suggesting less affinity for PAF substrate), and the Ile198Thr showed a six fold increase in Km. However, there was evidence for substantial Linkage disequilibrium (LD) between 198Thr/379Val and 92His/379Val (Kruse et al., 2000). In addition, Arg92His appears to be in complete negative LD with Ile198Thr (Ninio et al., 2004). Several other promoter and intron 1 SNPs (T-403C, C-209G, G+108ex1nt/T and T+107in1/C) have also been identified, although no functional data currently exists regarding these variants (Ninio et al., 2004). To this point, most of the polymorphisms listed above have been used to identify the potential associations of Lp-PLA2 with Asthma (PAF is implicated as a cause of disease)(Kruse et al., 2000; Rubin et al., 1987). Recent studies have shown that the variant alleles Thr198 and 379Val in particular are associated with atopic asthma in German and British Caucasians (Kruse et al., 2000).

Fig. 1.13: Schematic representing the mutated amino acids inherited by SNPs (Taken from (Karasawa et al., 2003)).



Mutated amino acids by inherited single nucleotide polymorphism

1.4.2.7 Lp-PLA2 role in atherosclerosis

Findings by the West of Scotland Coronary Prevention Study (WOSCOPS) group have raised questions about the potential role of Lp-PLA2 as a mediator of atherosclerosis (Packard et al., 2000b). Pro-inflammatory markers including CRP, fibrinogen and leukocyte count were used as predictors of CHD in middle aged men with hypercholesterolemia. All three markers, in addition to plasma Lp-PLA2 levels, were found to have a strong positive correlation with disease risk, but Lp-PLA2 was the only marker whose predictive ability was not compromised in multivariate analysis (Packard et al., 2000b). Both activity and mass of the enzyme in Caucasian populations has been shown to be a consistent risk marker for CHD, independent of traditional risk factors (Ballantyne et al., 2004; Blake et al., 2001; Blankenberg et al., 2003; Oei et al., 2005; Packard et al., 2000b; Sudhir, 2005; Winkler et al., 2005; Koenig et al., 2004). It

has also been suggested that CRP and Lp-PLA2 complement each other in identifying individuals at risk of CHD (Ballantyne et al., 2004).

Lp-PLA2 has also been implicated in diseases and populations often directly connected to atherosclerosis or inflammation, such as; asthma (Tsukioka et al., 1996; Stafforini, 2001), rheumatoid arthritis (Tselepis et al., 1999; Dulioust et al., 1992), acute myocardial infarction (Serebruany et al., 1998), sepsis (Graham et al., 1994), crohn's disease (Kald et al., 1996), ischemic stroke (Satoh et al., 1992), myocardial infarction (Ostermann et al., 1988), Tangier disease (Pritchard et al., 1985), diabetes mellitus (Hofmann et al., 1989), essential hypertension (Satoh et al., 1989), and peripheral vascular disease (Ostermann et al., 1987). However, some studies have also exhibited contradictory results. In particular, the Women's Health Study (WHS) demonstrated in multivariate analysis that there was no significant association between Lp-PLA2 levels and CHD risk after adjustment for several other risk factors including cholesterol (Blake et al., 2001). Although this could have been masked by the side effects of Oestrogen in those women taking hormone replacement therapy (estrogen lowers Lp-PLA2 expression (Miyaura et al., 1991)).

There is also the question of whether these studies confirm a functional role for Lp-PLA2 in atherosclerosis. Those studies investigating an association of Lp-PLA2 levels/ activity and risk suggest that Lp-PLA2 is a robust and reliable marker of atherosclerosis, particularly in men. However, in order to determine the functional role of this enzyme it is essential to consider confounding and reverse-causation when investigating causality. As with the case of CRP (section 1.2.2.6), functional genetic variants and/or specific inhibitors of Lp-PLA2 function would be helpful in confirming the relationship of this enzyme with atherosclerosis (To the authors knowledge there are currently no mouse knock-out models with which to investigate the atherosclerotic effects of Lp-PLA2 enzyme in an *in vivo* system).

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1.4.2.8 Lp-PLA2: Anti-Atherosclerotic?

Lp-PLA2 plays its primary anti-atherogenic role in the degradation of PAF and oxidised phospholipids. PAF is synthesised at sites of endothelial injury, and accumulates in atherosclerotic plaques, suggesting a role in the pathophysiology of this disease (Tselepis and John, 2002). PAF has been implicated in platelet activation, increased blood viscosity, smooth muscle cell formation, and oxygen free radical formation by macrophages; all of which contribute to, or exacerbate atherosclerosis (Tselepis and

John, 2002). PAF enhances the production of elastase by macrophages which in turn erodes the elastin of the fibrous cap, de-stabilising the plaque (Brocheriou et al., 2000). It also activates actin polymerisation, adhesion, and de-granulation in Neutrophils through its G-coupled receptor (Tjoelker and Stafforini, 2000). PAF is known to be stimulated by thrombin, bradykinin, histamine, and leukotrienes (Yamada et al., 2000); mediators present at high concentrations at sites of endothelial dysfunction. Recombinant Lp-PLA2 studies have provided some evidence to support the anti-inflammatory nature of this enzyme. Tjoelker *et al.* (Tjoelker and Stafforini, 2000) have demonstrated the ability of Lp-PLA2 to block PAF mediated events such as neutrophil polarisation and spreading in culture. Recombinant Lp-PLA2 also destroyed the ability of mast cell-derived PAF to stimulate serotonin release by platelets (Nakajima et al., 1997). Morgan *et al.* investigated vessel ligation ischemia/ reperfusion models: ligation of the large branch of the marginal coronary artery in New Zealand white rabbits for 45 min, followed by 2 hours of reperfusion resulted in coronary tissue necrosis, neutrophil infiltration, and compromised regional contractility. Administering recombinant Lp-PLA2 intravenously 15 minutes before reperfusion significantly reduced infarct size, hastened regional ventricular recovery and reduced neutrophilic infiltration of the reperfused area (Morgan et al., 1999).

Lp-PLA2 may also be protective due to its association with lipoproteins. Phosphatidylcholine in LDL particles, when exposed to copper mediated oxidation, generates fragmented alkyl phosphatidylcholine molecules with similar activities to PAF (Marathe et al., 1999). Indeed, PAF may contribute significantly to the biological activity of oxidised LDL (Androulakis et al., 2005). As well as a PAF function, Lp-PLA2 is able to hydrolyse oxidatively damaged phospholipids that may have accumulated within LDL particles (Stremler et al., 1991; Stremler et al., 1989). When depleted of Lp-PLA2 activity, LDL, IDL, and VLDL lipoproteins exhibited increased stimulation of monocyte chemotaxis and adhesion (Lee et al., 1999). The oxidative resistance of isolated LDL sub fractions was also reduced upon the administration of Lp-PLA2 inhibitors (McCall et al., 1999).

HDL associated Lp-PLA2 may also have important anti-atherogenic properties (Noto et al., 2003): Lp-PLA2 in HDL protects against the production and activity of minimally modified LDL (MM-LDL) by facilitating hydrolysis of active oxidised phospholipids to lysophospholipids, destroying the biologically active lipids in MM-LDL (Watson et al., 1995). The activity present on HDL could also reduce oxidative stress, prevent leukocyte recruitment to the intima, and progression of the lesion (De Geest et al.,

2000; Theilmeier et al., 2000; Quarck et al., 2001). This removal of potent biological mediators, may in part be due to another HDL associated enzyme, PON1 (Shih et al., 1998). Purified PON when exposed to PAF in the presence of the specific Lp-PLA2 inhibitor, SB-222657, is capable of hydrolysing PAF to lyso PAF and acetate (Rodrigo et al., 2001). Other work has quantified the contribution of non-Lp-PLA2 enzymes to the breakdown of PAF, to around 5% (Macphee et al., 1999). However, *in vitro* work has also shown that Lp-PLA2 is the sole oxidised phospholipid hydrolase of HDL when using calcium chelators that do not affect Lp-PLA2 activity (Marathe et al., 2003).

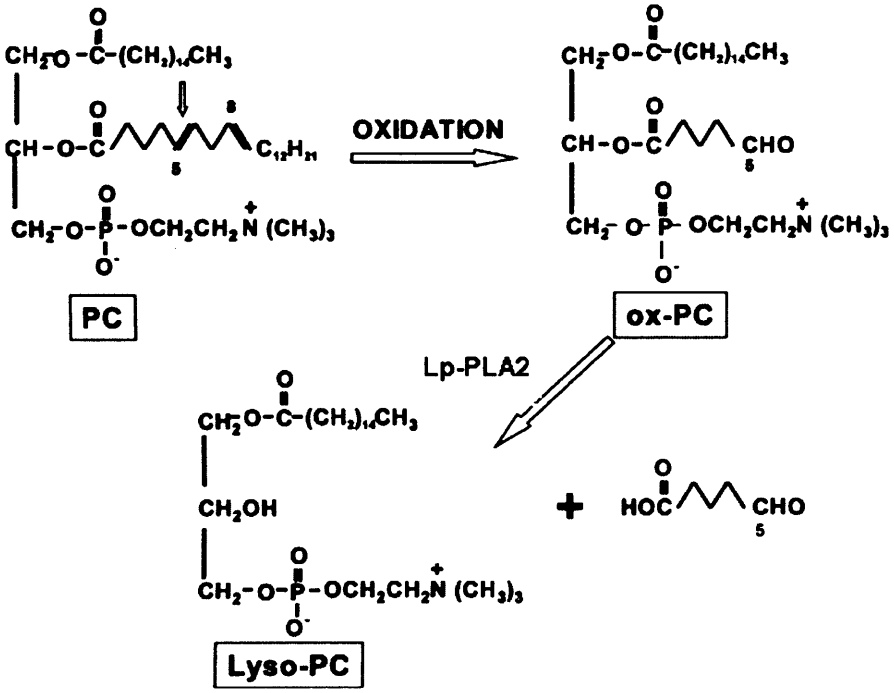
1.4.2.9 Lp-PLA2: Pro-Atherosclerotic?

In vitro work has shown that Lp-PLA2 when hydrolysing oxidised phosphatidylcholine and other short sn-2 acyl chain phospholipids, produces significant amounts of Lyso-PC and non-esterified fatty acids (Fig. 1.14)(Macphee et al., 1999; Tselepis and John, 2002). Research has also demonstrated that virtually all the Lyso-PC generated in oxidised LDL can be accounted for by Lp-PLA2 (Tew et al., 1996). Lyso-PC has been implicated in the promotion of monocyte recruitment, either by functioning as a chemo-attractant itself (Quinn et al., 1988), or via induction of endothelial leukocyte adhesion molecules (Kume et al., 1992). Lyso-PC has also been identified as the component of oxidised LDL that promotes both smooth muscle, and macrophage proliferation, induces endothelial dysfunction and is involved in the antigenicity of oxidised LDL (Chai et al., 1996; Sakai et al., 1996; Cowan and Steffen, 1995; Wu et al., 1998). The generation of oxidised free fatty acids (FFA) have related properties to Lyso-PC, but they are also an important precursor in eicosanoid production which themselves have several important metabolic effects. Some of these effects can be detrimental and may help the progression of atherosclerosis (Balsinde et al., 2002; Massiera et al., 2003). Indeed, PLA2 enzymes in general are a major source of Arachidonic Acid (AA)(Balsinde et al., 2002) (Fig. 1.11), which in turn is responsible for cell signalling processes through the prostaglandin pathway. AA itself has been implicated in inflammatory gene up regulation (Oestvang et al., 2004), adipocyte differentiation (Massiera et al., 2003) and skeletal muscle growth (Baracos, 2000).

In support of this pro-atherosclerotic argument, Hakkinen *et al.* (Hakkinen et al., 1999) have found increased Lp-PLA2 mRNA levels in human atherosclerotic lesions. In addition, ~6 fold higher Lp-PLA2 activity was detected in the atherosclerotic aortas of Watanabe heritable hyperlipidemic rabbits compared with normal aortas from control animals. Studies have also indicated the presence of Lyso-PC within atherosclerotic lesions (Portman and Alexander, 1969; Keaney, Jr. et al., 1995). Deposition of LDL

within the arterial wall is crucial and is dependent on the oxidative modification of LDL. Croft *et al.* (Croft et al., 1995) isolated LDL from 45 healthy individuals and studied Lp-PLA2 activity within the parameters of oxidation. Lp-PLA2, though not related in any way to LDL oxidation lag time (effectively a measure of oxidative resistance) was significantly associated with rate, and total diene production. Another aspect of Lp-PLA2 and its association with LDL is the cytotoxic effects on macrophages by oxidised LDL. Inhibition of Lp-PLA2 activity by SB 222657 (a specific inhibitor) has been shown to dramatically decrease the levels of Lyso-PC generated from oxidised PC, and diminished the ensuing toxicity and apoptosis of human monocyte-macrophages when the LDL was oxidised (Carpenter et al., 2001). In this experiment, Lp-PLA2 contributed over a third of the cytotoxic and apoptosis inducing effects of oxidised LDL seen (Lyso-PC concentrations were under the toxic threshold concentration therefore non-esterified fatty acids may be involved) (Carpenter et al., 2001).

Fig 1.14: Upon oxidation PC becomes a substrate for Lp-PLA2 generating lyso-PC and free fatty acids (Taken from (Tselepis and John, 2002)).



Work by our laboratory has shown for the first time that the altered activity *PLA2G7* 379V variant is associated with a lower risk of MI within a large European case control study (Abuzeid et al., 2003). Homozygosity for the V379 allele (rare allele frequency of 0.24) was associated with lower risk of MI, (Odds Ratio (OR) 0.56, 95%CI 0.32-0.98) compared to AV and AA individuals, and was maintained after adjustment for lifestyle factors and levels of inflammatory risk factors (C-reactive protein, fibrinogen, IL-6) (OR

0.46, 0.22-0.93). This may suggest that Lp-PLA2 is in some way pro-atherosclerotic (due to the reduced affinity for PAF substrate seen in the 379V form of the enzyme (Kruse et al., 2000)), supporting previous data from that found Lp-PLA2 activity associated with higher risk of CHD [reviewed in (Sudhir, 2005)]. Indeed, by showing that a functional polymorphism in the Lp-PLA2 gene affects risk, we have demonstrated that this enzyme is not just a marker, but potentially a modulator of disease as well. In support of this Ninio *et al.* have investigated the *PLA2G7* A379V polymorphism, as well as several other known non-synonymous and promoter variants, in the Athero*Gene* study of 1318 CAD patients and 485 controls. Analyses indicated that the effect of the A379V variant was independent of all the other polymorphisms. In support of our data, the 379V allele was associated with a reduction in CHD risk. However, the 379V allele was also associated with a weak but significantly higher Lp-PLA2 activity, suggesting a protective effect of Lp-PLA2 with regards to atherosclerosis. It is clear that further investigation is needed to determine the precise nature of this variant with regards to phospholipid substrate specificity and altered activity (Ninio et al., 2004). This PhD has aimed to further define the precise role of this enzyme with atherosclerosis.

1.3.3 Secretory Phospholipase A2, sPLA2 (*PLA2G2A* and *PLA2G5*)

1.4.3.1 General Properties

Two other candidate genes from the PLA2 family; *PLA2G2A* and *PLA2G5* have successfully been cloned and expressed from their closely linked chromosome position 1p34- 1p36.1 (Fig. 1.15), and exhibit properties similar to those PLA2 enzymes found in snake venom (Kramer et al., 1989; Seilhamer et al., 1989a; Seilhamer et al., 1989b). Both enzymes share structural and functional similarities such as a low molecular weight, several disulphide bridges, a calcium-dependent catalytic mechanism (unlike Lp-PLA2), and a well conserved three-dimensional structure (Dennis, 1994).

Both secretory PLA2 group IIA and V are small proteins in the region of 14KDa. The protein for IIA is encoded by a six exon sequence while V is encoded by 5 exons. Another difference is in the number of cysteine residues present in the two enzymes, 14 in group IIA and only 12 in V (Han et al., 1998). This confers a rigid structure, and resistance to pH and to thermal and proteolytic denaturation. Secretory PLA2 IIA has 23 cationic residues, Arginine and Lysine, which contribute to the enzymes high positive charge (pI=10.5). Specific cationic residues on its surface, among them Arg-7, Lys-10, and Lys-16, contribute significantly to the interfacial adsorption of the enzyme to the surface monolayers of cell membranes, lipoproteins, and aggregated phospholipids (sPLA2 has similar LDL/HDL binding preferences to Lp-PLA2) (Snitko et al., 1997). Furthermore, the positively charged regions may facilitate interaction with the negatively charged sulphated glycosaminoglycans of proteoglycans on cell membranes and extracellular matrix (Hurt-Camejo et al., 2001).

The biological functions of these two enzymes are still very much under investigation. Elevated plasma activities in inflammatory diseases as well as recent experimental findings suggest that the enzymes play important roles at certain stages of inflammation. Upon bacterial infection the two enzymes are involved in the primary host defence, mainly because sPLA2 (IIA and V) is able to destroy the lipid membranes of gram-positive bacteria (Kramer et al., 1989). The presence of considerable sPLA2 activity in tears and seminal plasma also suggests an antibiotic function (Qu and Lehrer, 1998). However, sPLA2 cannot directly degrade intact phospholipid membranes of gram-negative bacteria or cells. It appears that there needs to be some kind of disruption of the tightly packed lipid membrane exposing negatively charged phospholipids (termed 'flip-flop') (Niessen et al., 2003; Jaross et al., 2002). Other experimental data suggest that sPLA2 may contribute to the removal of 'injured' and

apoptotic cells and to the generation of eicosanoids, in particular arachidonic acid (AA) (Balsinde et al., 2002). Secretory PLA2 enzymes are also known to aid in lipid digestion, release of potent lipid mediators in response to cytokine stimulus, cell proliferation, phospholipid repair, lipoprotein catabolism, and tumourigenesis (Hurt-Camejo et al., 2001).

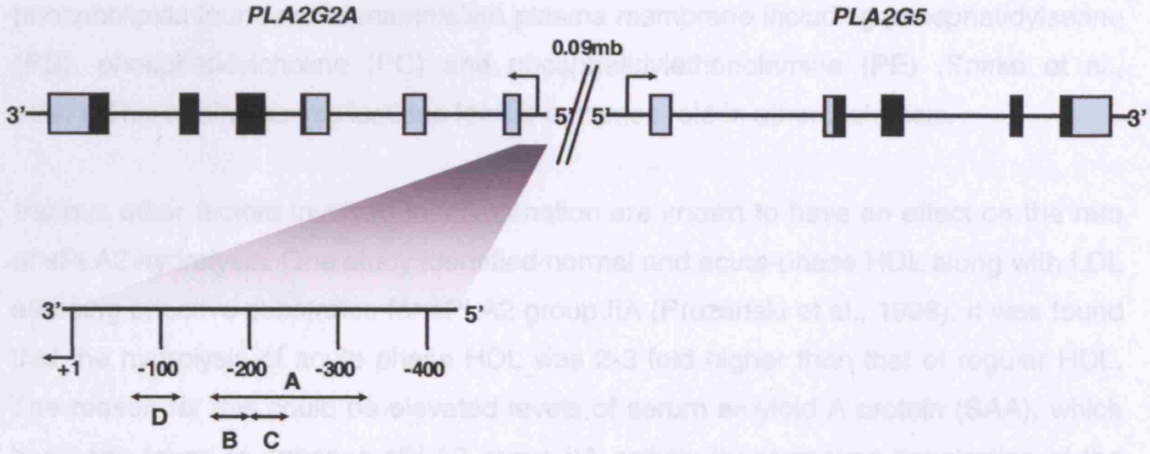
1.4.3.2 Expression and transcriptional regulation of sPLA2

Secretory PLA2 enzymes are constitutively expressed in tissues dealing with inflammatory responses, such as the spleen, thymus, tonsils, liver, and bone marrow. However, sPLA2 can also be found in prostate, cartilage, placenta, and lacrimal glands (Jaross et al., 2002). Smooth muscle cells, endothelial cells, platelets, mast cells, neutrophils, macrophages, and hepatic cells are the major cellular sources (Jaross et al., 2002). The secretion of sPLA2 by inflammatory cells and hepatocytes is induced by the pro-inflammatory cytokines IL-1, IL-6, TNFα, and IFNγ (Peilot et al., 2000; Nakano et al., 1990). Both sPLA2 enzymes are acute phase reactants with plasma activities being low in healthy humans, and stimulated to high levels in response to inflammation (Jaross et al., 2002).

Both *PLA2G2A* and *PLA2G5* genes are in a negative orientation on the chromosome and 90Kb apart, suggesting that the promoters are controlled by similar elements (Fig. 1.15). *PLA2G2A* has been more extensively studied and provides better information about its promoter regulation. The promoter sequence in human (Kramer et al., 1989; Seilhamer et al., 1989b) shows several proximal C/EBP transcription factor binding sites [-125;85] critical for the up-regulation of expression in HepG2 cells in response to IL-1 and IL-6 (Fan et al., 1997; Paradon et al., 1998; Andreani et al., 2000) (Fig 1.15; region D). The region -326 to -176 (Fig. 1.15 region A) as a whole, represses gene expression under basal conditions (Massaad et al., 2000). However, the region -210 to -176 5' of the transcription start site appears to be critical for IL-1β up-regulation and also contains potential C/EBP transcription binding sites (Massaad et al., 2000) (Fig. 1.15; region B). Massaad *et al.* also identified a region -247 to -210 that contains an insulin response element (IRS) and a NF-1 like transcription binding site (Massaad et al., 2000) (Fig 1.15 region C). By contrast, in the rat promoter, several other areas of the proximal promoter region have been identified as C/EBP transcription binding sites. The rat promoter also has an NF-kB site which is not present in humans (Antonio et al., 2002). In humans, the low basal levels of sPLA2 and strong induction of expression by IL-6 (and other Interleukins) could well be mediated by the numerous C/EBP

transcription factors identified, since this transcription factor family is stimulated by IL-6 (Andreani et al., 2000).

Fig. 1.15: Orientation and organisation of the *PLA2G2A* and *PLA2G5* genes on chromosome 1. Black boxes represent translated gene. There are no other genes in the 90kb missing region. Within the promoter region A [-326;-176] has been found to suppress overall activity. Region B [-210;-176] contains potential C/EBP binding sites and appears critical for IL-1,6 up-regulation. Region C [-247;-210] contains a potential Insulin response element and a NF-1 binding site. Region D [-125;-85] represents a strong positive proximal promoter element containing C/EBP



1.4.3.3 Substrate preferences of sPLA2

In broad terms sPLA2 IIA and V have a similar substrate requirement to that of Lp-PLA2, except they have a calcium dependency and a limited role in the degradation of PAF. They are able to hydrolyse phospholipids at the sn-2 position, yielding free fatty acids (FFA) and lysophospholipids. Through the remodelling pathway (Fig. 1.11), these products can lead to the formation of pro-inflammatory mediators such as; PAF, eicosanoids, and Lysophosphatidic acid. In addition, polyunsaturated FFA are susceptible to free radical mediated oxidation and can further contribute to cytokine mediated responses. However, the specificities of these two enzymes for phospholipid species are very different, leading to confusion about secretory PLA2 activity *in vivo*. Previous *in vitro* work has found that Group V sPLA2 is able to bind to and hydrolyse PC and phosphatidylethanolamine (PE) membranes twice as efficiently as group IIA (Group IIA sPLA2 is not as efficient at binding the bulky cationic head group of PC and PE)(Han et al., 1998), thereby enabling it to hydrolyse the most common phospholipid species present in lipoproteins and cell surface membranes (Kim et al., 2000). Since

most cell membranes are constructed from PC, it is conceivable that Group V is highly regulated to prevent long-term tissue and organ damage.

By contrast, sPLA2 IIA is closely involved in the generation of Lysophosphatidic acid (LPA). This is a potent lipid second messenger which stimulates platelet aggregation, cell proliferation, smooth-muscle contraction and monocyte activation (Fueller et al., 2003; Ninio, 2005). The PLA2-catalysed hydrolysis of phosphatidic acid (PA) is thought to be a primary synthetic route for LPA (Fourcade et al., 1995). Kinetic analysis of recombinant sPLA2 IIA has demonstrated that it prefers PA as a substrate over other phospholipids found in the mammalian plasma membrane including phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Snitko et al., 1997). This again has implications for the enzymes role in atherosclerosis.

Various other factors involved in inflammation are known to have an effect on the rate of sPLA2 hydrolysis. One study identified normal and acute-phase HDL along with LDL as being effective substrates for sPLA2 group IIA (Pruzanski et al., 1998). It was found that the hydrolysis of acute phase HDL was 2-3 fold higher than that of regular HDL. The reason for this could be elevated levels of serum amyloid A protein (SAA), which has been found to enhance sPLA2 group IIA activity by promoting penetration of the dense lipid monolayer (Pruzanski et al., 1995). Other *in vitro* work has demonstrated that increasing levels of ceramide progressively enhanced sPLA2 activity by altering the liposome membrane structure. Sphingomyelin (SPH) was found to reverse this, and is known to confer stability onto lipid bilayers (Koumanov et al., 2002). Indeed, the addition of sphingomyelinase (SMase) produces asymmetry in lipid bilayers and makes the lipid more susceptible to sPLA2 activity (Fourcade et al., 1995).

1.4.3.4 Secretory PLA2 role in atherosclerosis

1.4.3.4.1 Modification of Lipoproteins and retainment in the arterial wall

The following observations regarding secretory PLA2 are confined to experiments involving the IIA enzyme, unless otherwise specified. However, it is reasonable to assume that sPLA2 V will also share many of these properties. Secretory PLA2 is capable of hydrolysing phospholipids on both HDL and LDL (Jaross et al., 2002). However, some conditions are able to significantly alter the rate of hydrolysis; one particular study showed that minimally modifying LDL by copper oxidation, led to a 25% increase in LDL susceptibility to hydrolysis. Further 'ageing' of the LDL at 37°C for an hour led to a 26-fold increase in phospholipid hydrolysis (Eckey et al., 1997).

Other modulators of sPLA2 activity with regards to lipoproteins appear to be SPH, and Proteoglycans. SPH mediates several effects; it is able to competitively bind to the sPLA2 active site, and prevent hydrolysis of other phospholipid species, thereby reducing activity (Koumanov et al., 1997). It is also known to confer structural rigidity and oxidative protection to lipoproteins. Removal of SPH by sphingomyelinase (SMase), decreases the lag time and rate of copper induced lipid peroxidation in LDL, by its effect on lipid fluidity and packing density (Subbaiah et al., 1999). LDL is unable to fuse or aggregate under normal physiological conditions without first being modified (Hakala et al., 1999; Hakala et al., 2001). When sPLA2 was added to LDL *in vitro*, it was able to cause aggregation of LDL, but not fusion (Hakala et al., 1999). On the addition of SMase, LDL rigidity was removed and the particles fused; a process that occurs in atherosclerotic plaque formation, and is critical to the generation of foam cells (Oorni et al., 1998). Proteoglycans on the other-hand may increase sPLA2 activity, and increase LDL binding. Sartipy *et al.* have successfully demonstrated that sPLA2 interacts with proteoglycans via their glycosaminoglycan (GAG) moiety (Sartipy et al., 1996).

Most of the products produced from the action of sPLA2 on lipoproteins are successfully transferred to albumin. However, experiments *in vitro* have demonstrated that more than half the products stay linked with lipoproteins (Gorshkova et al., 1996). There is a significant increase in free cholesterol on the surface of the lipoprotein, (Gorshkova et al., 1996) and the negative net charge increases, resulting in increased electrophoretic mobility (Kleinman et al., 1988; Menschikowski et al., 1995c). The treatment of LDL and HDL *in vitro* with sPLA2 has also shown that with decreasing PC content, specific epitopes are exposed. In LDL, Kleinman *et al.* (Kleinman et al., 1988) demonstrated that modified LDL bound to neutrophils in a non-specific manner, independent of the presence of FFA and Lyso-PC, possibly mediated by Apo-B 100. Similar findings have been demonstrated with Apo-AI in HDL (Menschikowski et al., 1995a), with one study demonstrating that HDL (Apo-AI) has altered catabolism and uptake after modification (Tietge et al., 2000). LDL, when modified by immobilised sPLA2, formed smaller, more dense particles that had a preference for GAG. This may in part be due to a change in the organisation of the surface structure of Apo-B100, exposing GAG binding regions, and therefore increasing the possibility of phagocytosis and retention in the arterial intima (Sartipy et al., 1999) (table 1.6).

Experimental data suggests that all sPLA2-modified lipoproteins have an enhanced ability to transfer cholesterol to vascular macrophages as well as to non-vascular

tissues such adipose cells; a mechanism that is regulated in some part by the degree of hydrolysis of phospholipids (Natarajan et al., 1990). If uptake is primarily by macrophages within the arterial wall, the process will result in foam cell formation and the progression of atherosclerosis (table 1.6).

Table 1.6: Summary of Lipoprotein modification by secretory PLA2

Modifications of Lipoprotein	**Properties of sPLA2 modified Lipoproteins**
Decreased particle size	LDL interaction with GAG and retention in arterial wall
Increased density	Enhanced Foam cell formation and LDL uptake
Altered Apolipoprotein conformation	Increased concentrations of FFA, Lyso-PC, and free cholesterol on surface
Increased –ve charge	Lowered antioxidant potential of HDL (Leitinger et al., 1999)

1.4.3.4.2 Secretory PLA2 in the arterial wall

There is convincing evidence that sPLA2 localises to atherosclerotic plaques. A strong sPLA2 immunoreactivity has been observed in the arterial media in atherosclerotic vessels (Hurt-Camejo et al., 1997). Romano *et al.* (Romano et al., 1998) used Electron Microscopy (EM) to show a stronger sPLA2 immunoreactivity in the arterial intima of atherosclerotic tissue compared to non-atherosclerotic tissue. EM-immunogold examination revealed that the majority of sPLA2 was localised along the extracellular matrix, associating with collagen fibers and other extracellular matrix structures. Intracellular PLA2 was observed in electron-dense vesicles in intimal cells, and sPLA2 was also found in contact with large, extracellular lipid droplets. Raised monoclonal antibodies against sPLA2 have been used to immuno-stain healthy arteries, which proved to be totally negative for sPLA2. Diseased arteries showed localisation of the enzyme to SMC, macrophages, extra-cellular matrix areas and to regions close to the necrotic core of the plaque (Menschikowski et al., 1995b).

1.4.3.4.3 Summary of sPLA2 involvement in atherosclerosis

Studies concentrating on murine models have found group IIA in particular to be associated with atherosclerotic plaque formation. Transgenic mice over-expressing sPLA2 group IIA from macrophages resulted in significant increases in the extent of atherosclerosis in aortic arches. There was no localisation of PLA2 in any other areas of the vessel wall (Webb et al., 2003). Ivandic *et al.* (Ivandic et al., 1999) showed that transgenic mice (6 fold higher expression of human sPLA2 group IIA) exhibited dramatically increased atherosclerotic lesions when maintained on a high-fat, high-cholesterol diet. Surprisingly, the transgenic mice also had significant atherosclerotic lesion formation when maintained on a low-fat chow diet. The transgenic mice exhibited decreased levels of HDL and slightly increased levels of LDL compared with non-transgenic littermates. Epidemiological studies show that circulating levels of sPLA2 IIA have been found to be higher in CHD patients and those with angina, compared to controls (Kugiyama et al., 1999; Kugiyama et al., 2000; Liu et al., 2003). Circulating sPLA2 IIA levels have also been associated with increased future risk of CHD, independent of CRP, in prospective analysis (Boekholdt et al., 2005a). Recent work has also shown that sPLA2 IIA mass levels correlate with measures of insulin resistance (HOMA IR) in T2DM individuals (Leinonen et al., 2003). Very little genetic data is currently available concerning either *PLA2G2A* or *PLA2G5* genes and their potential association with atherosclerosis. Indeed, the only genetic study to date investigating familial adenomatous polyposis and sporadic colorectal tumours found no functionally significant variants in the *PLA2G2A* gene region (Tomlinson et al., 1996).

In essence, sPLA2 IIA and V may exert a pro-atherogenic effect by three main mechanisms shared with those of Lp-PLA2 (summarised in Fig 1.16):

1. Release of high local concentrations of non-esterified free fatty acids (NEFA), oxidised NEFAs, and lyso-phospholipids, which may affect the function of a range of vascular cells at the sites of LDL accumulation. These products may either act as intracellular second messengers or can be further metabolised into pro-inflammatory lipid mediators like eicosanoids (Balboa et al., 1996), PAF and LPA (Snitko et al., 1997). Lyso-PC itself is chemo attractant for monocytes and T-lymphocytes (Asaoka et al., 1993), induces the expression of growth factors and adhesion molecules in endothelial cells, is mitogenic for macrophages and vascular smooth muscle cells (Pruzanski et al., 2001), inhibits endothelium-dependent relaxation and endothelial-cell motility. Lyso-PC also alters sub-endothelial heparin sulphate proteoglycan making it more adhesive to

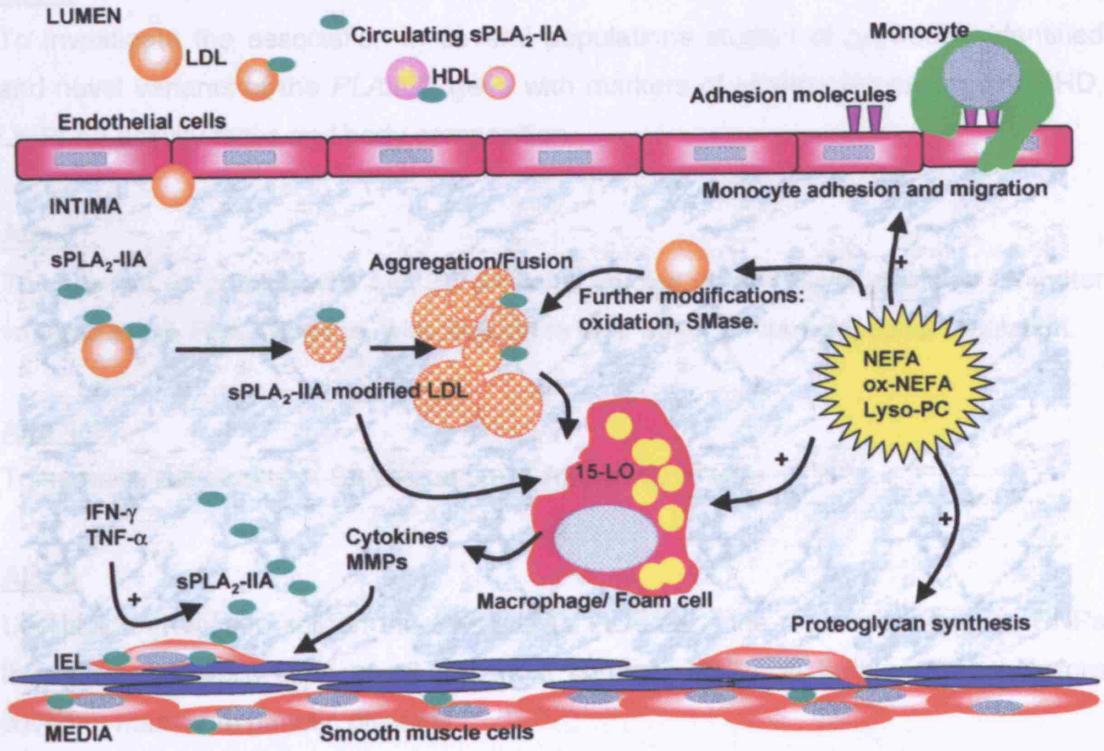
monocytes. Beside these effects, there is evidence that the extracellular generation of lyso-PC by secretory PLA2 may promote tissue inflammation and haemostatic disturbances (Hurt-Camejo and Camejo, 1997).

2. Secretory PLA2 may modify LDL to the smaller and denser atherogenic form, and increase retention in the arterial cell wall. Within the intima, LDL is susceptible to enzymic modification (SMase and lipoxygenase) and oxidation.

3. The enzyme actively contributes to aggregation and fusion of proteoglycan-bound LDL while decreasing the anti-oxidative potential of HDL (Hurt-Camejo et al., 2000; Oorni et al., 1998).

Secretory PLA2 enzymes have yet to be associated with any significant anti-atherogenic properties. This may be due to the different substrate preferences of sPLA2 and Lp-PLA2, in particular the degradation of PAF.

Fig 1.16: Schematic of the pro-atherogenic actions of sPLA2 in the arterial intima (courtesy of (Hurt-Camejo et al., 2001))



1.5 Aims of PhD

Hypothesis

Lipoprotein associated phospholipase A2, and the secretory PLA2 enzymes are thought to mediate inflammation, oxidative modification of LDL, and the generation of phospholipid-based cell signalling molecules; all of which are implicated in the progression of atherosclerosis. However, there is a need to clarify the relationship of these enzymes with regards to their causal role in the progression of atherosclerosis. The intention of this PhD was to determine whether common genetic variation in the secretory PLA2 *PLA2G2A/PLA2G5* genes and Lipoprotein-associated PLA2 gene *PLA2G7*, directly influenced individual differences in markers of atherosclerosis and risk of CHD.

Aim 1

To identify common polymorphisms in the proximal promoter of *PLA2G7* using Single Strand Conformation Polymorphism (SSCP) analysis.

Aim 2

To investigate the association in several populations studies of previously identified and novel variants of the *PLA2G7* gene with markers of atherosclerosis, risk of CHD, Lp-PLA2 activity/mass and body composition.

Aim 3

To carry out (in parallel with Aim 2) functional studies on any novel identified promoter variants in the *PLA2G7* gene, with regards to their effect on transcriptional regulation.

Aim 4

To examine the effects of Statins on Lp-PLA2 activity *in vitro*

Aim 5

Use bioinformatics to screen the *PLA2G2A / PLA2G5* gene regions for tagging SNPs that infer the allelic state of all the most common SNPs in both genes, therefore covering maximum genetic diversity.

Aim 6

Investigate the association of the inferred 'haplotypes' in *PLA2G2A / PLA2G5* with markers of atherosclerosis in a cross-sectional study.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and commonly used stocks

2.1.1 Reagents

DNA extraction: All reagents were supplied by Sigma-Aldrich Ltd. (Poole, UK)

Polymerase chain reaction (PCR): PCR oligonucleotides and *Taq* polymerase were supplied by Invitrogen Ltd (Paisley, UK). 50mM $MgCl_2$ supplied by Bioline (London, UK). All necessary restriction enzymes were supplied by New England Biolabs Inc (Hertfordshire, UK) or Roche Diagnostics (Lewes, UK). dNTPs were supplied by Pharmacia Biosystems Ltd (Milton Keynes, UK). 96 well PCR plates were obtained from Corning Inc. (Hemel Hempstead, UK). All other reagents supplied by Sigma-Aldrich Ltd. (Poole, UK).

Gels: 19:1 30% acrylamide:N,N'-methylenebenebisacrylamide was supplied by Protogel, National Diagnostics (Hull, UK). TEMED (NNN',N'-tertramethylethylethylenediamine and Ammonium persulphate (APS) were supplied by BDH (Leicestershire, UK). Agarose was supplied by Helena Biosciences (Sunderland, UK) and 10× Tris Borate EDTA (TBE) was supplied by Severn Biotech Ltd (Worcestershire, UK). 1kb ladder was supplied by Invitrogen Ltd (Paisley, UK).

Taqman assays: All Taqman assays were obtained from Applied Biosystems (California, USA). Taqman Absolute QPCR Rox mix and 384 well plates were supplied by ABgene (Surrey, UK).

Sequencing and SSCP: All oligonucleotides were obtained from Invitrogen Ltd (Paisley, UK). ABI Big Dye v3.1 5× sequencing buffer was obtained from Applied Biosystems (California, USA). Formamide loading dye was obtained from Amersham Biosciences Ltd. (Buckinghamshire, UK). Sequagel XR rapid sequencing solution and Sequagel Buffer reagent was obtained from National Diagnostics (Hull, UK). All other reagents were supplied by Sigma-Aldrich Ltd. (Poole, UK).

Cloning and Functional work: *Escherichia coli (E.coli)* strain DH5α™ competent cells, Macrophage Serum Free Medium (MSFM), heat-inactivated foetal bovine serum, 1× Phosphate Buffered Saline (PBS), oligonucleotides, RPMI-1640 growth medium,

Dulbecco's MEM medium, OptiMEM and trypsin-EDTA were purchased from Invitrogen Ltd (Paisley, UK). THP-1, HL-60 and Huh-7 cell lines were purchased from the European Collection of Cell Cultures, ECACC (Salisbury, UK). The pRL-TK and pGL3-Basic reporter vector, ligase buffer, dual luciferase assay kit, and passive lysis buffer were purchased from Promega (Southampton, UK). Ficoll paque and GFX PCR DNA and Gel band purification kit was supplied Amersham Biosciences Ltd. (Buckinghamshire, UK). Simvastatic acid was kindly donated by Elisabeth Teissier (Pasteur institute- Lille, France). T-25/T-75 cell culture flasks and Nunclon 96 well cell culture plates were purchased from Nunc (Roskilde, Denmark). Quikchange site-directed mutagenesis kits were purchased from Stratagene (California, USA). QIAprep spin miniprep kit, Effectene Transfection kit and RNeasy RNA extraction mini kit were supplied by Qiagen (Crawley, UK). Lipofectamine 2000™ transfection kit was supplied by Invitrogen Ltd (Paisley, UK). Gene Elute HP maxiprep kit, Bacteriological-Agar, Tryptone, Yeast extract, Sodium Chloride (NaCl) and all other reagents were supplied by Sigma-Aldrich Ltd.(Poole, UK).

RT-PCR: SUPERSCRIPT II reverse transcriptase, ribonuclease H (RNase H), random hexamer oligonucleotides, 10mM dNTPs and all relevant buffers were purchased from Invitrogen (Paisley, UK). Probes and oligonucleotides were supplied on demand from Applied Biosystems (California, USA).

2.1.2 Commonly used stock solutions

L-Agar: 950 ml of distilled water (dH_2O); 10g of Bacto-tryptone; 5g of Bacto-yeast; 10g of NaCl; 20g Bacto-agar

L-Broth: 950 ml of distilled water (dH_2O); 10g of Bacto-tryptone; 5g of Bacto-yeast; 10g of NaCl.

Ammonium persulphate solution (APS): 0.25g APS dissolved in 1ml distilled water.

MADGE Loading Dye: 0.0015% bromophenol blue; 0.015% xylene cyanol; 10% glycerol; 10mM EDTA

NH_4 polmix buffer: 16mM $[NH_4]_2$ SO_4; 67mM Tris-HCL pH8.4; 0.01% Tween 20; 2mM dATP; 2mM dTTP; 2mM dGTP; 2mM dCTP

Sticky Silane: 0.5% v/v glacial acetic acid; 0.5% v/v γ methacryloxy-propyl-trimethoxy-silane

TBE buffer: 0.04M Tris-borate; 1mM EDTA pH7.4

Reagent A (sucrose lysis buffer): 0.32M sucrose; 5mM $MgCl_2$; 10mM Tris-HCl ph7.5; 1% Triton-X-100

Reagent B (nuclei lysis buffer): 10mM Tris-HCl pH8.2; 0.4M NaCl; 2mM Na_2EDTA pH 8.0

TE Buffer: 10mM Tris-HCl; 1mM EDTA pH 7.6

2.2 Genotyping studies

2.2.1 DNA extraction from whole Blood by the 'Salting Out Method'

DNA was extracted from 5mls of potassium-EDTA or citrated anti-coagulated peripheral blood using a salting out method (Miller et al., 1988). Before starting the extraction process (performed in batches of 24 samples), all samples were carefully logged and entered into a database with a unique identifier in order to preserve the anonymity of individuals in the study. The DNA extraction process involves several steps, cellular and nuclear lysis, de-proteinisation, extraction and precipitation, as detailed below.

Cell and nuclear lysis

Blood samples were thawed and transferred to a labelled 30ml polypropylene tube. Cold (4°C) sucrose lysis buffer (15ml) was added to each tube and mixed by hand inversion. Tubes were centrifuged at 4°C for ten minutes in batches of 12 at 1800g (Sorvall RC5 centrifuge using rotor SA-600). The supernatant was carefully discarded without disturbing the pellet and 2ml of reagent A (Sucrose lysis buffer) was added and the pellets re-suspended using a disposable pastette for each sample. A further 18ml of reagent A was added before invert-mixing once more. Samples were then centrifuged again for a further ten minutes. Following centrifugation, the supernatant was discarded, and 2ml of reagent B (nuclear lysis buffer) was added and the pellet re-suspended with a pastette.

De-proteinisation

Using a 10ml pipette, 1ml of 5M sodium perchlorate was added and the sample inverted. Samples were then left on a shaker for fifteen minutes.

DNA extraction

Two millilitres of cold chloroform (-20°C) was added to each tube, using a glass 25ml pipette and mixed by hand. Samples were then centrifuged at 1800g for three minutes at room temperature. Following addition of cold chloroform, the DNA from each sample partitioned into the upper aqueous phase within each tube. This upper phase was then carefully transferred into a fresh, labelled 30ml polypropylene tube, without disturbing the organic phase.

Precipitation and washing

Ten millilitres of 100% cold ethanol (stored at -20°C) was poured slowly down the side of each tube to precipitate the DNA. These were then sealed and gently mixed. DNA from each tube was then gently "spooled" using a sterile pastette. Samples were washed in 70% ethanol before being transferred into a sterile labelled microtube containing 1ml Tris-EDTA (TE) buffer. At this final stage, care was taken to ensure that the identifier on the tube corresponded with the hospital number and patients name recorded on the microtube label.

Handling DNA samples that fail to spool

Occasionally, no DNA was seen to precipitate after the addition of 100% ethanol. These samples were placed in the freezer overnight at -20°C. They were then centrifuged at 1500g for fifteen minutes, before decanting the supernatant to leave a small DNA pellet. Tubes were allowed to air dry before adding 0.5ml of TE buffer. At this stage samples were left overnight at 37°C before re-suspending and transferring the contents into labelled microtubes.

Dissolving the DNA

Sealed microtubes were placed in an incubator at 37°C overnight, before being placed in a cold cabinet (at 4°C) for storage. Following DNA extraction, samples were left for a period of at least four weeks to allow the DNA to completely dissolve. At this stage a series of "stock" and "working" 96-well arrays were generated.

Generating Array Sheets

In order to facilitate a high-throughput genotyping technique - microtitre array diagonal gel electrophoresis (MADGE)(Day et al., 1995); it was firstly essential to generate a clear and accurate array sheet listing the samples by code number within an 8 x 12 grid (Fig. 2.1).

Fig. 2.1: An example of a standard array grid

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	26	34	42	50	58	66	74	82	90
B	2	10	18	27	35	43	51	59	67	75	83	91
C	3	11	19	28	36	44	52	60	68	76	84	92
D	4	12	20	29	37	45	53	61	69	77	85	93
E	5	13	21	30	38	46	54	62	70	78	86	
F	6	14	22	31	39	47	55	63	71	79	87	
G	7	15	23	32	40	48	56	64	72	80	88	
H	8	16	24	33	41	49	57	65	73	81	89	

2.2.2 Manual Standardisation of DNA arrays

This was performed to standardise all DNA samples to 15ng/µl. The steps are described below.

Diluting the samples 1 in 10 for the plate reader

Gloves and filtered tips were used throughout. 10µl of each sample was pipetted out into the corresponding well of a 96 well Costar UV plate. Wells 12E, 12F, 12G and 12H were kept empty as blanks (100µl dH_2O). Using a manual Eppendorf 300, 8 channel pipette, 90µl of dH_2O was added to each well and mixed by re-suspending the sample. The plate was then centrifuged (Sigma 4-15) at 200g for thirty seconds, before measurement in the plate reader.

Measuring the absorption at 260nm and 280nm

This was performed using the Tecan GENios plate reader utilising the Magellan 3 software package. After gently wiping the bottom of the plate with a tissue, the plate was placed into the reader. The 260/280nm filter slide was inserted into the excitation port. The corresponding absorption at 260 and 280nm was then recorded for each well in the plate and exported to an excel file.

Calculation of the volumes for manual array standardisation

The volume of DNA required (added to 750µl of dH_2O) to achieve a concentration of 15ng/µl was calculated using an Excel programme. If any of the DNA volumes were above 200µl, then the volume was halved and 375µl of dH_2O added to these wells. If any of the DNA concentrations were near 15ng/µl, the DNA was transferred neat.

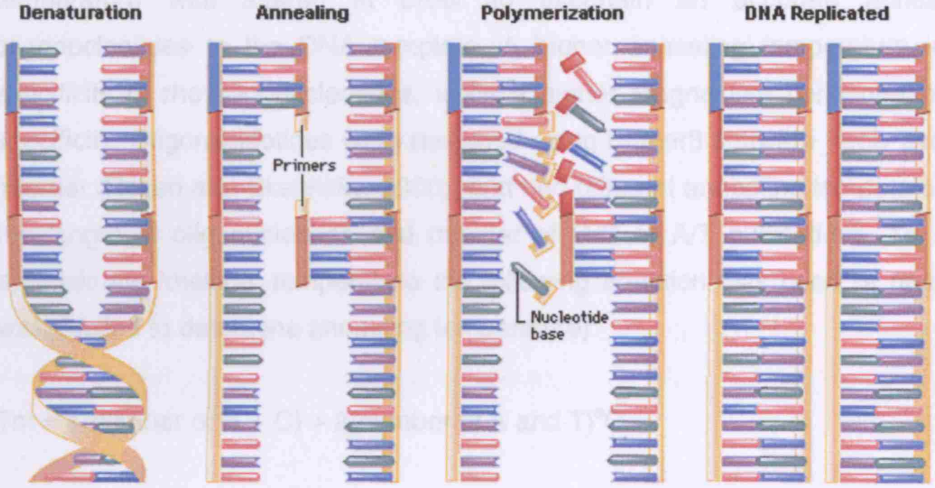
Creating Stock and Working Arrays

Stock arrays were created in labelled 96-well Beckman's array. These were stored at -20°C. To create working arrays, 100µl of each sample was removed from the stock array, and transferred to another labelled 96-well array, before storing at 4°C.

2.2.3 Polymerase chain reaction & Restriction Digest

The technique of PCR relies on double stranded DNA being denatured into single strands by heat, consequently annealing with oligonucleotides, and with the addition of DNA polymerase and nucleotide bases, the synthesis of a double strand on cooling (Mullis et al., 1986). This process leads to binary replication, generating large quantities of DNA in a short period of time. The first step is therefore a short period of high temperature to denature or 'melt' the DNA. This is followed by cooling in the presence of oligonucleotides that are complementary to the DNA either side of the sequence to be studied. These oligonucleotides anneal, and a DNA polymerase adds nucleotides base by base, thus replicating the DNA (Fig. 2.2). The polymerase used is derived from the bacterium *Thermus aquaticus* (*Taq*) and is heat stable. Therefore, it does not need to be replenished after each cycle of heating and cooling.

Fig. 2.2: Schematic representation of the polymerase chain reaction (PCR). Denaturation of double stranded DNA occurs at 95°c (melting). Annealing of oligonucleotides varies according to the relative amounts of the four bases present. Polymerisation with *Taq* usually occurs at 72°c (polymerisation).



2.2.3.1 Sample Preparation for Polymerase Chain Reaction

Following extraction and dispensing into working 96-well arrays, DNA samples were prepared for PCR by centrifuging the DNA working-array at 200g for one minute. This was to ensure that all the DNA dilutions were at the bottom of their respective wells, reducing the possibility of cross-well contamination when the array lid was removed. Two and a half microlitres of each sample (45ng/µl of DNA) was then removed from each array and transferred into a standard 96-well PCR plate from Corning Inc. (Hemel Hempstead, UK) using a Finnipipette multichannel dispenser (Life Sciences, Basingstoke, Hants, UK). Positive and negative controls were utilised to ensure accuracy. Extreme care was taken to ensure that samples were placed in the identical orientation as in the original arrays. Loaded plates were then centrifuged at 200g for thirty seconds to ensure that the DNA was at the bottom of each well, and then dried on a Thermal Cycler block (MJ Tetrad DNA Engine Thermocycler) at 80°C for ten minutes.

A bulk mix of reagents was made up for each PCR, allowing adequate volume for the planned number of reactions, with an additional 10% added to ensure that the mix would not run short. PCR oligonucleotides and *Taq* polymerase were kept on ice and added just before the commencement of the reaction. PCRs were performed in a total volume of 20µl made up with distilled water. Each reaction contained 1x concentration of polmix [50mM

KCl, 10mM Tris-HCl (pH 8.3), 0.2mM dATP, dGTP, dTTP and dCTP], $MgCl_2$, 8pmol of each oligonucleotide and 0.4U of *Taq* polymerase. Prior to the genotyping of whole studies, an optimisation procedure was conducted; magnesium concentration and temperature was altered in order to ascertain an accurate annealing of the oligonucleotides to the DNA template. A higher annealing temperature increases the specificity of the oligonucleotides, while a higher Magnesium concentration lowers the specificity. Oligonucleotides were designed using Primer3 software freely available on the internet (Rozen and Skaletsky, 2000), and had different annealing temperatures based on the length of oligonucleotide and number of G/C or A/T nucleotides. To ascertain the approximate 'melting' temperature the following equation was used (a reduction of 4°C was applied to determine annealing temperature):

Tm = 4(number of G + C) + 2(number of A and T)°C

The PCR mix was added to each well of the PCR plate using an automatic Biohit repeating dispenser (Alpha Laboratories, UK). Each sample was overlaid with 20µl of mineral oil to prevent evaporation. The microtitre plate was then sealed with a clear sticky plastic lid and carefully labelled. Plates were centrifuged at 200g for thirty seconds to ensure good mixing of the reaction components in each well. PCR amplification was performed on an MJ Tetrad DNA Engine Thermocycler. After an initial DNA 'melting' stage of 95°C for 5 minutes, the following cycle conditions were used for all PCRs:

1. 95°C for 30 seconds (s)
2. A variable annealing temperature for 45s (Table 2.1)
3. 72°C for 30s

The three steps were repeated for 30 to 35 cycles (depending upon individual optimisations), followed by a final 'polymerisation' step of 72C for 10 minutes. Table 2.1.A summarises the oligonucleotide sequences and PCR conditions used in this thesis.

2.2.3.2 Restriction digestion

Restriction enzymes are derived from bacteria, and cleave double stranded DNA at a particular sequence. The enzyme translocates along the DNA until a particular recognition site is reached, where the DNA is cut. The restriction enzyme is sensitive even to a single base change in the recognition sequence, and thus can be used to detect point mutations

and single base polymorphisms. A single base change can either eliminate or create a cutting site for a particular enzyme.

A restriction enzyme digest mix with the recommended buffer system was made up in a 1ml Eppendorf tube on each occasion, containing sufficient enzyme (0.3- 3 units) to digest the PCR products in each well of the PCR plate: in a typical digest mix for a 96 well plate, 130μL of Buffer was added to 200 units of enzyme and made up to 500μL with distilled water. 5µl of digestion mix was then added to 8µl of each reaction product using a repeater pipette as for the PCR mix. Each omniplate was then centrifuged at 200g for thirty seconds to ensure that the PCR product and restriction enzyme mixed well. The PCR/digestion mix was then incubated overnight at the recommended temperature. The specific conditions and possible product sizes for each genotyping assay are detailed in table 2.1.B.

Table 2.1: A summary of the PCR and digest conditions used for genotypes under study. Standard 95°c melting and 72°c extension temperatures were used.

A

Gene variant	Oligonucleotides	$MgCl_2$	Annealing temperature	Number of cycles	Fragment length
PLA2G7 A379V (T1136C)	FOR 5'-AGGGAGACATAGATTCAACTG-3'	2mM	54°C	30	69bp
rs1051931	REV 5'-CGTTTTGTAAGAATGCTAATGAA-3'				
PLA2G7	FOR 5'-ACTTTGTCTTCACCTTTGTCTTA-3'	2.5mM	57°C	35	138bp
G-1230A **rs13210554**	REV 5'-ATGCCCTACCAAAGACATCTGTAA-3'				
PLA2G5	FOR 5'-GGACTGTTGATGGTGGGAGT-3'	1.5mM	61°C	35	186bp
C1640T **rs640022**	REV 5'-CCAGGTATGATGGTGCACAG-3'				

B

Gene variant	Enzyme and units (U) per well	Buffer system	Manufacturer of Enzyme and buffer system	Incubation temperature	Fragment sizes produced
PLA2G7	*PstI*	NEB buffer 3	New England Biolabs	37°C	69/49/20bp
A379V	2U				
PLA2G7 G-	*MaeIII*	MaeIII 2xbuffer	Roche Diagnostics	55°C	138/114/24bp
1230A	0.3U				
PLA2G5	*PvuII*	NEB buffer 2	New England Biolabs	37°C	186/166/20bp
C1640T	3U				

2.2.4 Detection of DNA

2.2.4.1 Agarose gels

In order to check the successful amplification and size of PCR products, agarose gels were utilised. For a 2% gel, 2 grams of agarose (Helena Biosciences, Sunderland UK) was mixed with 100ml of 1×TBE solution containing 10μL of Ethidium bromide (10μg/μL). A microwave oven was used to heat the mixture and dissolve the agarose at 50°C. The melted agarose was poured into a plastic gel tray (10 × 14cm) and a comb inserted. For bigger DNA products, lower percentage gels were made by reducing the amount of agarose dissolved in the TBE solution (1g agarose = 1% gel). Solid gels were placed into an electrophoresis tank containing 750ml of 1xTBE buffer solution. 2µl of MADGE loading dye was added to 5μL PCR product and the entire volume was mixed thoroughly and placed in the separate, submerged wells of the gel. 2μL of a 1Kb ladder (Invitrogen, Paisley UK) was pipetted into the central well in order to size relevant products. All agarose gels were run at 100 volts (v) for a minimum of 30 minutes.

2.2.4.2 Microtitre Array Diagonal Gel Electrophoresis (MADGE)

The DNA fragments produced by restriction enzyme digest were separated by electrophoresis on a non-denaturing polyacrylamide gel, using Microtitre Array Diagonal Gel Electrophoresis (MADGE)(Day et al., 1995). This technique makes it possible to electrophorese all the 96 wells of a standard PCR plate on a single gel, by allowing the samples to run diagonally. Use of MADGE allowed the 96 well DNA array format to be retained throughout the screening process. Standard 7.5% MADGE gels were made up in batches for economy of time.

MADGE consists of an open arrangement of 8x12 wells each 2mm deep. The wells are arranged at an angle of 71.2° to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used (Fig. 2.3 A). Thus the maximum track length is 26.5mm allowing sufficient travel for genotype resolution.

Before making the mix, glass plates of appropriate size (160 x 100 x 2mm) were rigorously cleaned and hand dried. 5 drops of γ-methacryloxypropyltrimethoxysilane ('sticky' silane) were spread across the plates and left to air-dry. Silane was used to ensure that the MADGE gel would adhere to the glass plates. The MADGE mix was then made up accordingly (volume for 6 plates) in a conical flask: 25ml of 10×TBE solution, 62.5ml of

19:1 acrylamide:N,N'-methylenebenebisacrylamide, 162.5ml of distilled water, and 900μL of TEMED.

Polymerisation was initiated by the addition of ammonium persulphate (as the combined solution begins to set within thirty seconds of mixing), before the solution was mixed and quickly poured into the three-dimensional former. A glass plate was then gently placed over the mould (silane side facing downwards) taking care not to trap any air bubbles. This was then left for fifteen minutes to set, using a small weight to ensure that the glass did not slip whilst the gel was setting. Excess gel was trimmed from the edges of the MADGE former before the glass plate and attached gel were then prized away from the plastic former. MADGE gels were stored in a plastic Stuart box containing 500ml 1xTBE solution.

Gel staining and loading

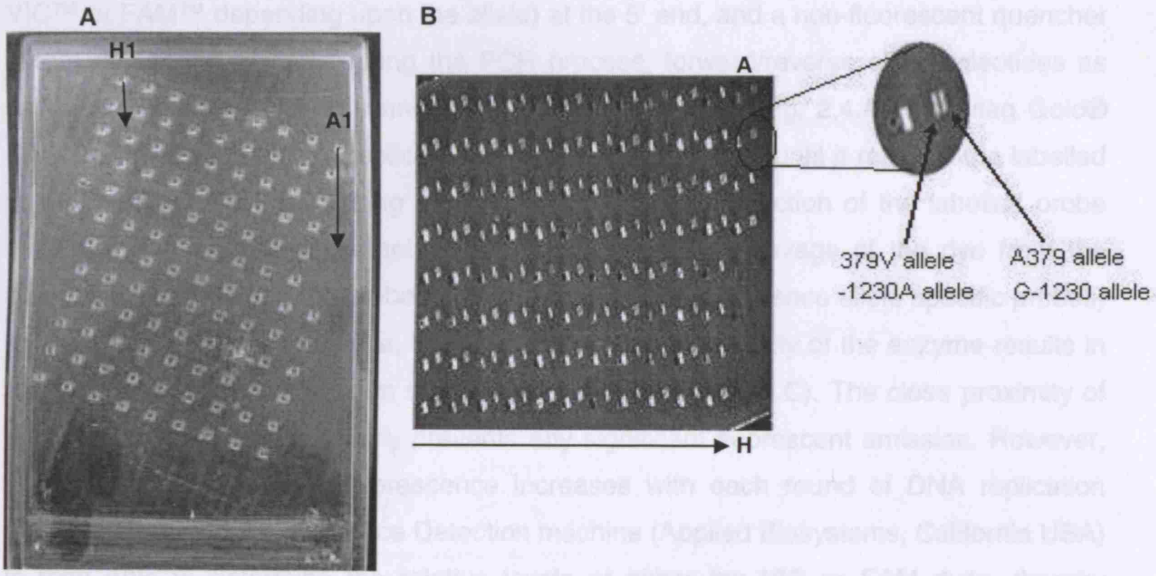
Prior to loading a gel with digested PCR product, each gel was stained with Ethidium Bromide (EtBr). This was achieved by placing them individually in a Stuart box, shielded from direct light, containing 100ml of 1x TBE and 10µl EtBr for 20 minutes.

Whilst the gels were being stained, the PCR-digest product was prepared for loading onto the MADGE gel. 2µl of MADGE loading dye was added to each well of a new, round-bottomed, loading tray, followed by 5µl of each digested sample, using a multi-channel pipette to pick up the samples from under the oil in the plates. The digested samples were gently mixed, by aspirating the formamide dye–digest mixture up and down several times into the pipette, before dispensing them on the digest plate.

After placing the stained MADGE gel into an electrophoresis tank containing 750ml of 1xTBE buffer solution, a multi-channel pipette was used to transfer 5µl of this digest/dye mixture to the wells of the gel. At all times the samples were kept in the same layout as on the PCR tray, allowing each sample to be easily identified without being re-labelled. The gel was electrophoresed at 150v for a minimum of 30 minutes.

Following electrophoresis, the gel was viewed and photographed under ultraviolet light using the UVP Gel Documentation System. Care was once more taken to ensure the correct orientation of the MADGE under UV. Figure 2.3 B illustrates the typical pattern obtained for the *PLA2G7* polymorphisms genotyped.

Figure 2.3: Perspex former (A) and visualised MADGE gel (B) for the *PLA2G7* A379V and G-1230A gene variants. Perspex former is used to create angled indentations in the gel. Both wild type genotypes were cut by their respective enzymes. Arrow at the bottom of the gel represents the direction of electrophoresis.



2.2.5 Genotyping quality control

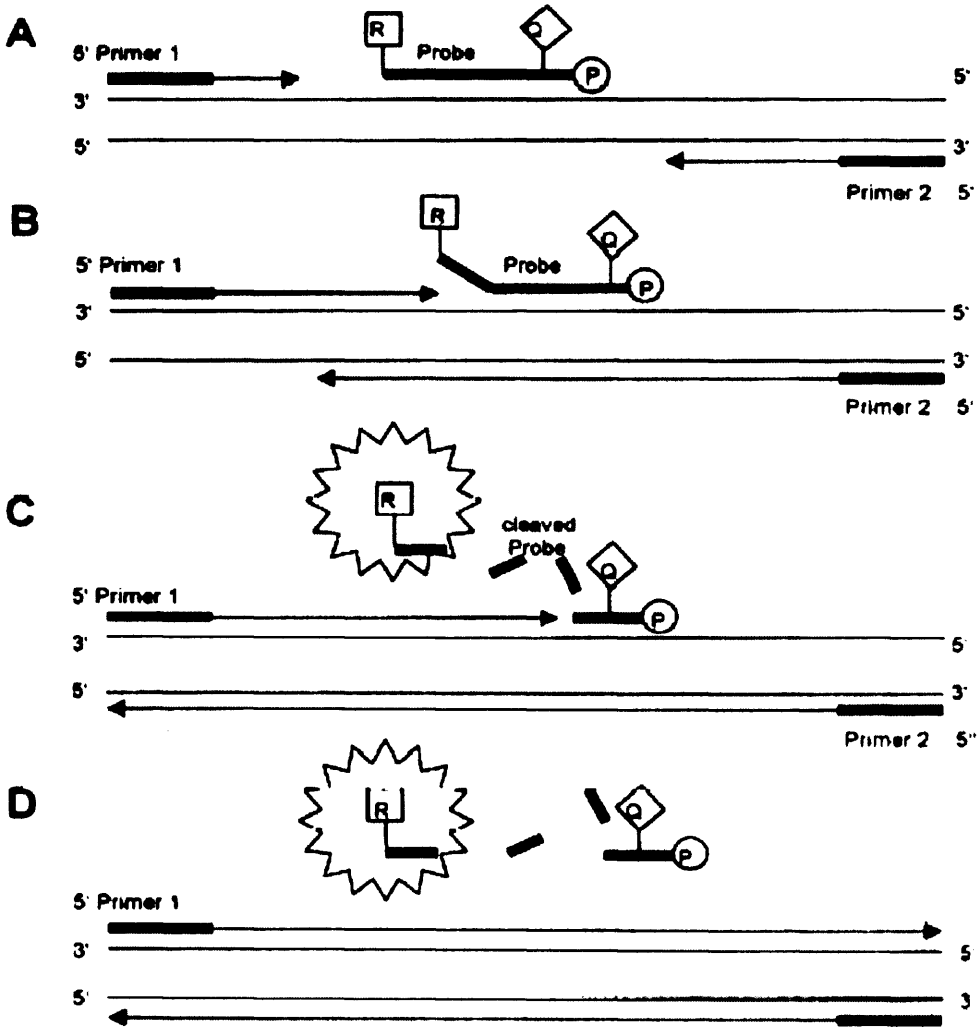
All genotyping was performed in a double blind fashion using both positive and negative controls. The results were rechecked by two individuals at the time of MADGE imaging and during data entry into the computer database. Any apparent genotype differences were resolved by repeat PCR. Overall there was excellent reproducibility with >95% consistency between samples.

2.2.6 Taqman® genotyping

Taqman® assay technology

The principle behind the Taqman® reaction is described in Fig. 2.4. The method involves the inclusion of two fluorescent, dye-labelled probes for each allele of a specific variant. The allele specific probes each contain a short sequence of DNA, a reporter dye (Labelled VIC™ or FAM™ depending upon the allele) at the 5' end, and a non-fluorescent quencher (NFQ) dye at the 3' end. During the PCR process, forward/reverse oligonucleotides as well as the labelled probes, anneal to the DNA of interest (Fig. 2.4.A). Amplitaq Gold® DNA polymerase is able to replicate the single strand of DNA until it reaches the labelled probe. Any non-specific binding results in a weakened interaction of the labelled probe with the DNA and displacement of the intact probe (no cleavage of the dye from the quencher)(Fig.2.4.B). If the probe is entirely complementary (hence allele specific probes) to the annealed DNA sequence, the 5' to 3' exonuclease activity of the enzyme results in the cleavage of the 5' dye from the rest of the probe (Fig. 2.4.C). The close proximity of the dye to the quencher usually prevents any significant fluorescent emission. However, once the dye is cleaved, fluorescence increases with each round of DNA replication (Fig.2.4.D). A 7900HT Sequence Detection machine (Applied Biosystems, California USA) is then able to determine the relative levels of either the VIC or FAM dyes, thereby determining the specific genotype.

Fig. 2.4: Schematic of the Taqman® assay system. A. represents the annealing of the fluorescent probes and oligonucleotides. B. If the fluorescent probe is not identical to the DNA sequence, the probe is displaced by the Taq. C. Successful annealing leads to 5'-3' exonuclease of the probe. D. The VIC™ and FAM™ labels fluoresce and are picked up by the Taqman® machine.

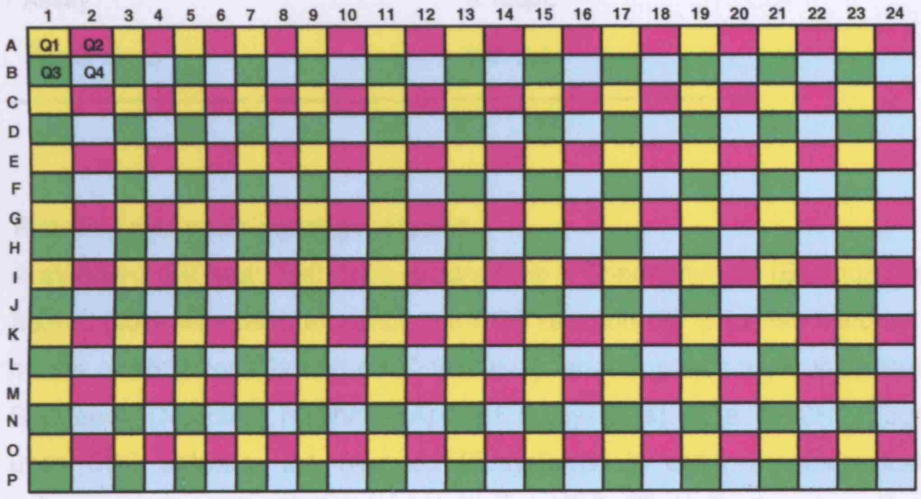


Preparing the DNA

In contrast to traditional PCR and MADGE based technology, the Taqman® system enables high throughput genotyping in a 384 well format. In order to use this system, DNA was first standardised to an optimal concentration of 1.25ng/μL using the same methodology described in section 2.2.2. A Biomek 2000 robot (Beckman-Coulter, High Wycombe UK) was used to aliquot 4μL of standardised DNA from a 96 well stock array into a 384 well plate (5ng total) with 16 wells left blank to act as negative controls. A data

sheet was also compiled in order to identify each well to patient ID number (Fig. 2.5). The plates were dried out overnight at room temperature in sterile paper bags and stored until use.

Fig. 2.5: Schematic of a 384 well plate. Each colour represents an individual 96 well plate as pipetted by the Beckman robot.



Taqman master mix

Forward/reverse oligonucleotides and labelled probes were ordered using the 'Assay by demand' service available on the Applied Biosystems website (www.appliedbiosystems.com). Table 2.3 lists all the assays successfully designed by Applied Biosystems for this thesis. In addition, all the relevant rs numbers for each SNP are listed. For each 384 well plate, a master mix was made of the ABgene QPCR Rox mix, the individual assay mix, and distilled sigma water (Table 2.2). 4μL of mix was then applied to each well of the 384 plate using a manual Eppendorf 300, 8 channel multi-dispensing pipette and centrifuged (Sigma 4-15) at 200g for thirty seconds. A clear plastic lid (ABgene, Surrey UK) was applied to seal the plate.

Table 2.2: Assay reagents for a 384 well plate master mix. 4μL was carefully pipetted into each well of the 384 well plate using an 8 channel multi-pipette

Reagents for a 40x assay mix	**Volume in μL per well**
Taqman Absolute QPCR Rox mix	2.5μL
Assay	0.125μL
Sigma dH_2O	2.375μL

Reading and entering of genotypes

A standard two step heat cycle program on a Thermohybaid (Basingstoke, UK) 384 well heated block was used to initiate the PCR reaction (95°C for ten minutes, followed by 40 cycles of 95°C for 15s and 60°C for 1min), and all plates were then read on a 7900HT Sequence Detection machine (Applied Biosystems). The 7900HT Taqman® machine uses SDS software 2.1 (Applied Biosystems) in order to differentiate the different genotypes (Fig. 2.6). SDS 2.1 produces an allelic discrimination plot as well as assigning genotypes automatically to an excel file containing patient ID numbers according to array position. To ensure no incorrect inputting of data, a second researcher validated all the genotypes before the data was finally entered into the analysis database.

Fig. 2.6: Screen shot of a typical allelic discrimination plot. The three different coloured dots represent each individual genotype: Blue and red represent homozygotes with the green dots representing heterozygotes. Light blue crosses show the negative controls. Those dots which were not tightly clustered (circled in the figure) to a particular group were re-genotyped.

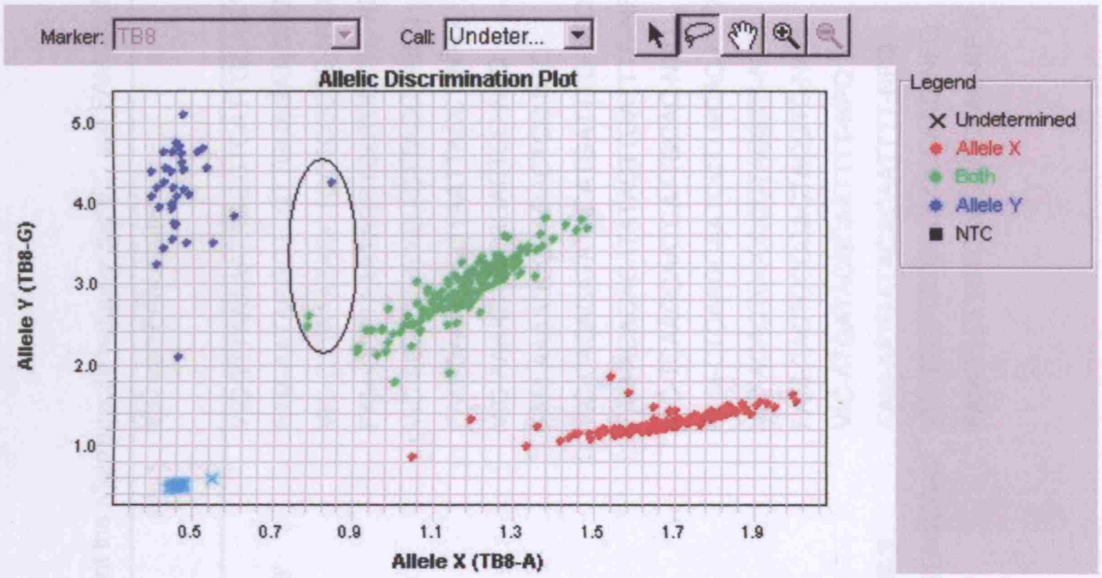


Table 2.3: Taqman assays design for all SNPs. Highlighted bases represent the allele specific nature of the VIC and FAM labelled probes.

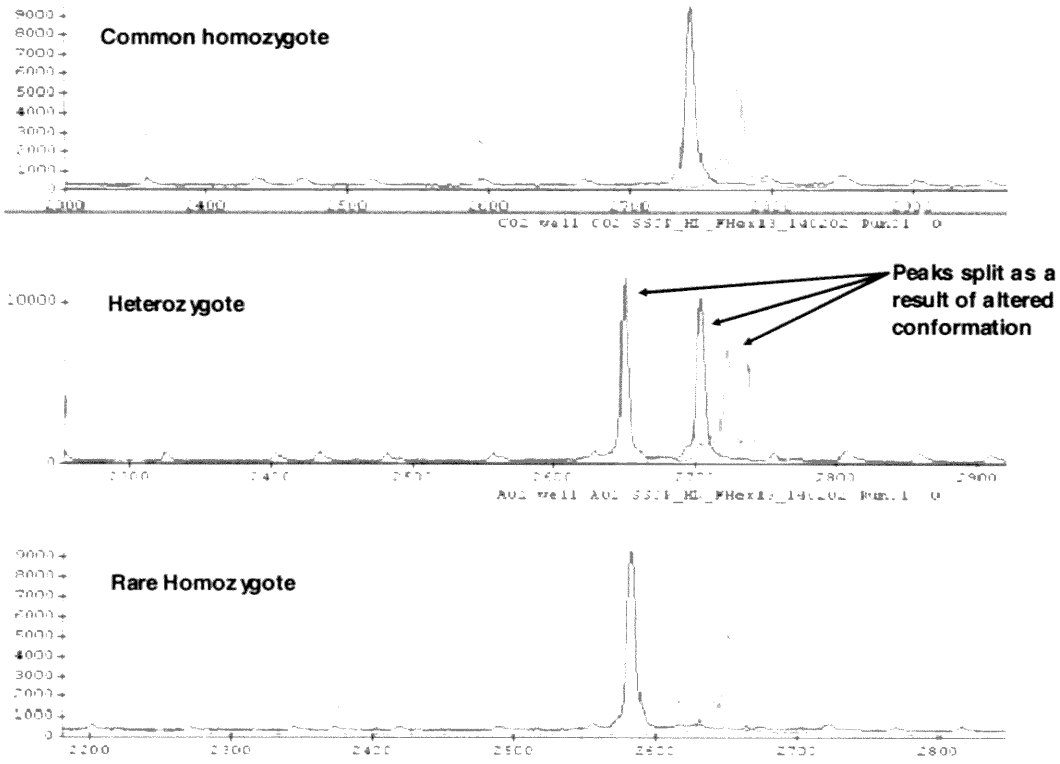
Gene, SNP and rs number	Forward and Reverse oligonucleotides	VIC and FAM probes	Genotype
PLA2G2A T-655C	FOR 5'-CTGCCCTAGTGATCTCCTCTTAACT-3'	VIC-TTTAAAAATAGGGTC**A**TTGCAGAT-NFQ	T=VIC
rs1774131	REV 5'-TCCCGGTACTCTTAGAATGTGATCTT-3'	FAM-AATAGGGTC**G**TTGCAGAT-NFQ	C=FAM
PLA2G2A C763G	FOR 5'-CAGCCTTGTGCCTCACCTA-3'	VIC-CTTAAATAGCT**G**CTCCCTC-NFQ	G=VIC
rs11573156	REV 5'-CAGGCCGTCTTGTTTGTTCTG-3'	FAM-CCTTAAATAGCT**C**CTCCCTC-NFQ	C=FAM
PLA2G2A G1022T	FOR 5'-GTGTAAGAGAGGATGTTGGCACTAT-3'	VIC-ACACTCTCGTA**C**TTACCT-NFQ	G=VIC
rs3753827	REV 5'-GTTAACACACCTATGCCCACATACA-3'	FAM-CACTCTCGTA**A**TTACCT-NFQ	T=FAM
PLA2G2A G1983A	FOR 5'-CCCCAGACAGCTGACTCATG-3'	VIC-AAAAGCGT**G**GACTCC-NFQ	G=VIC
rs2236771	REV 5'-CTTAAAGGACTCAGGCCATGGG-3'	FAM-AAAAGCGT**A**GACTCC-NFQ	A=FAM
PLA2G2A T4892A	FOR 5'-CAACTCCGTGCTTAACCAAAGAAG-3'	VIC-CACACATATAC**A**TGATTTG-NFQ	A=VIC
rs876018	REV 5'-GCTAACAGTGCATAGGGCAATG-3'	FAM-ACACACATATAC**T**TGATTTG-NFQ	T=FAM
PLA2G2A T5128G	FOR 5'-GGGAGGCTGAGGCTGAA-3'	VIC-TCAAGCAATC**A**TTGCAC-NFQ	T=VIC
rs3767221	REV 5'-GCTGGCCTTGAACTTCTGTGA-3'	FAM-CTCAAGCAATC**C**TTGCAC-NFQ	G=FAM
PLA2G5 C-1437A	FOR 5'-TGGAAGCCGCTGATACAGAAG-3'	VIC-ACACTCCCC**C**CTACGTC-NFQ	C=VIC
rs11573185	REV 5'-GGCTCTGCTGTGTTGGAGAA-3'	FAM-CACTCCCC**A**CTACGTC-NFQ	A=FAM
PLA2G5 G-1251A	FOR 5'-GCTCAGTTGCACCAACAGCTA-3'	VIC-ATGATAC**G**CAATTTT-NFQ	G=VIC
rs2148911	REV 5'-TTTGTCAATTCTTCCCAATCTGATGAGT-3'	FAM-AATGATAC**A**CAATTTT-NFQ	A=FAM
PLA2G5 G-423A	FOR 5'-TGGATATAAAAACCATTATTGGGACAATAGGG-3'	VIC-TTCTTGTCCCC**G**CCAAG-NFQ	G=VIC
rs11573191	REV 5'-GACAGCCTGCTTAGAATCTTGACTA-3'	FAM-TTCTTGTCCCC**A**CCAAG-NFQ	A=FAM

Gene, SNP and rs number	Forward and Reverse oligonucleotides	VIC and FAM probes	Genotype
PLA2G5	FOR 5'-AGGTTGCAGTGAGCTGAGATC-3'	VIC-TTGCTCTGTTG**C**CCCAG-NFQ	Gins=VIC
Gins1742Gdel	REV 5'-GTGCTGAGTGTGTGTGTTTGG-3'	FAM-CTTGCTCTGTTGCCCAG-NFQ	Gdel=FAM
rs11573203			
PLA2G5	FOR 5'-GGAGGCCAACAGATCAAGAAATGA-3'	VIC-CCATTGAAAAGA**TA**GCTTGT-NFQ	TAins=VIC
TAins11069TAdel	REV 5'-GCCTTCTTCTGGGAGCTGTAAATA-3'	FAM-CCATTGAAAAGAGCTTGT-NFQ	TAdel=FAM
rs11573248			
PLA2G5	FOR 5'-GAGAACCATCAGGGAGGTCATC-3'	VIC-CTGTATGTTTC**A**CTAGCTTT-NFQ	T=VIC
T22507G	REV 5'-AGGGAGGCATTGCGATCTG-3'	FAM-TGTATGTTTC**C**CTAGCTTT-NFQ	G=FAM
rs622450			
PLA2G7	FOR 5'-GCTTTTGTAAGAATGCTAATGAAGCTTTGT-3'	VIC-TAAGATCAATAGCT**A**CATTTG-NFQ	A=VIC
A379V (T1136C)	REV 5'-ACACATGCTCAAATTAAAGGGAGACA-3'	FAM-ATCAATAGCT**G**CATTTG-NFQ	G=FAM
rs1051931			

2.3 Single strand conformation polymorphism (SSCP) analysis

SSCP is a method used to identify genetic variation in a single strand of DNA, typically between 150-500 nucleotides in length in a number of samples (Hayashi, 1991; Sheffield et al., 1993). By using oligonucleotides that overlap, it is possible to analyse large regions of DNA with complete coverage. Under non-denaturing conditions each individual strand will form a secondary structure unique to that particular sequence, and these conformational differences result in the DNA strands migrating differentially through a gel matrix. By using Fluorescent labelled oligonucleotides, it is possible to differentiate these conformational changes using a capillary based sequence detection system such as the MegaBACE™ 1000 (Amersham Biosciences, 96 capillaries, DNA sequencing and genotyping system). Fig 2.7 shows the typical peaks of fluorescence observed when SSCP is conducted on the MegaBACE™ 1000: each peak represents the point at which a labelled strand passes through the fluorescence detection beam of the capillary gel matrix. If the conformation is altered by the presence of a variant in the DNA sequence, then the peak will be split.

Fig. 2.7: A typical SSCP pattern observed for a common polymorphism when using MegaBACE genotyping programme. Green peaks represent size markers. Blue peaks are single stranded FAM labelled DNA, while red peaks represent HEX labelled DNA.



Eight pairs of overlapping Forward and reverse unlabelled oligonucleotides (Table 2.4) covering 2000bp (1800bp of which was 5' of the transcription start site) of the *PLA2G7* promoter were designed using oligonucleotide 3 software and optimised for PCR as described in section 2.2.3.1. PCR products were run on 1-2% agarose gels stained with Ethidium bromide along with a 1Kb ladder in order to confirm that the correct length of DNA had been amplified. After optimisation, the same oligonucleotides were ordered with the attachment of either a FAM (forward oligonucleotide) or HEX (reverse) dye label at the 5' of the oligonucleotide. Using the optimised PCR conditions set out for the unlabelled nucleotides, it was possible to generate labelled PCR products. PCR was run in a 96 well plate without any oil overlaying the 20μL reaction mixture (A heated lid program was used to prevent evaporation). For genetic variation analysis, DNA from 50 healthy individuals was amplified. PCR products were diluted 1 in 50 with sterile deionised water and 5μL of diluted sample was plated into a new 96 well omniplate. Heteroduplex (HD) analysis was carried out under the same running conditions as SSCP but without denaturing the PCR product. The diluted PCR product was denatured at 95 ^{o}C for 5 minutes and, to prevent strands re-annealing, immediately snap cooled on water/ice prior to loading. Samples were run on MegaBACE™ 1000. Samples were injected at 3kV for 45 seconds with electrophoresis performed at 10 kV for 75 minutes on a non-denaturing matrix (3 % linear polyacrylamide non-denaturing long read matrix, Amersham Biosciences) at 28^{o}C. Analysis was carried out using the MegaBACE™ genotyping programme (Genetic-Profiler v1.5, Amersham Biosciences).

Table 2.4: Overlapping oligonucleotide pairs used in SSCP analysis of the *PLA2G7* promoter region. Primer set 1 are defined as being the most distant from the transcription start site.

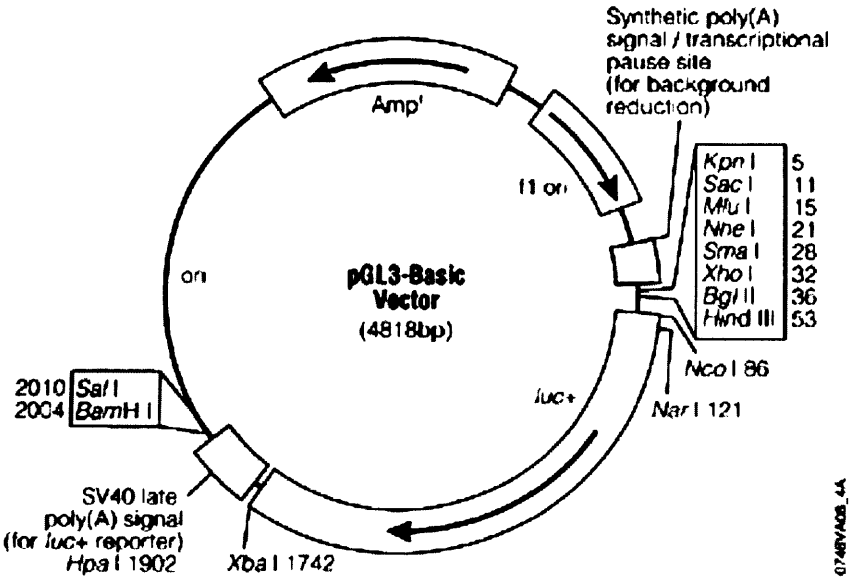
Primer set	Oligonucleotides	Mg^{2+}	Annealing temperature	Cycles	Fragment length (bp)
1	5' FAM-TTGATTGAAGGGAAGAGGTGAGAC-3'	2mM	59°C	35	259
	5' HEX-GGCTTCCTGTATTATATCTGGCAAC-3'				
2	5' FAM-TGTCTGTGTTGACTTATCCTCTTGG-3'	3mM	65°C	29	290
	5' HEX-AGTTGGCATTTGGCAGGTTAGT-3'				
3	5' FAM-TGGGCACTCTTCACTGGGCAT-3'	2.5mM	63°C	35	302
	5' HEX-GCCCCACAAATATCAATCTAAAAGGA-3'				
4	5' FAM-GACGTTAGAGATGTCTTTGGTAGGG-3'	2.5mM	66°C	35	335
	5' HEX-GCTTCTCGGTCTCCTCATCCATAAA-3'				
5	5' FAM-TGCATTTCGTGCTCACAAACTGC-3'	3mM	65°C	29	230
	5' HEX-TTAAATCGTGTTTTGGTTTTTGGTG-3'				
6	5' FAM-TAAGCAAAATTTCATCTCCAGTTCTG-3'	2.5mM	67°C	35	414
	5' HEX-TTTTCCGCACTCCGATACACTTTA-3'				
7	5' FAM-AGGGAGAAATGACACCAGCACAG-3'	2.5mM	69°C	35	454
	5' FAM-CTCTCCTCCCCTGGTGATGCT-3'				
8	5' FAM-ATGGTCTTGGGGAGGGTGCT-3'	2mM	63°C	35	478
	5' HEX-AGCGGGTGCGACTCAGCGA-3'				

2.4 Cloning

2.4.1 Cloning of the *PLA2G7* promoter into pGL3-Basic

A 1.9kb section of the *PLA2G7* promoter was generated by the PCR reaction described in section 2.2.3, and inserted into the pGL3-Basic reporter vector containing the Luciferase gene (LUC) and ampicillin resistance gene (selectable marker) as follows. The fragment of interest was amplified from genomic DNA using a pair of oligonucleotides that contained two restriction endonuclease recognition sites [Forward *(Mlu I)* 5'-CG**ACGCGT**TGCTGGCAATGAGAGAAGTG-3' and Reverse *(Bgl II)* 5'-GA**AGATCT**TCCGCCTCAAAAGAAAGAAA-3']. The PCR was modified in order to achieve a high fidelity amplification of the sequence: proof reading Platinum *Pfx* DNA polymerase (Invitrogen, Paisley UK) was used along with the manufacturers recommended conditions and buffer (2.5mM $MgCl_2$ and 57°C annealing temperature for 35 cycles). The 50μL PCR product was electrophoresed through a 1% agarose gel (stained with Ethidium Bromide) along with a 1Kb ladder in order to confirm the correct size of fragment. The band of interest was cut out using an ultra-violet (UV) lamp and a sterilised razor blade, and purified using the GFX Gel band purification kit according to manufacturers instructions (Amersham Biosciences Ltd., Buckinghamshire UK). The pGL3-Basic reporter vector contains a polyclonal site with several endonuclease recognition sequences (Fig. 2.8): 300ng of the pGL3-Basic vector, and 50μL of the purified PCR product were incubated with *Bgl II/MluI* restriction enzymes in the appropriate buffer system for one hour. Ligation of the vector and PCR fragment was performed by mixing 100ng of vector and 15ng digested PCR product with 1μL ligase and 1μL ligase buffer (New Englannd Biolabs, Hertfordshire UK). The reaction was incubated for 4 hours at room temperature. In order to check that the PCR fragment had successfully ligated into the vector, two flanking oligonucleotides to the polyclonal site were designed in order to sequence the region (section 2.5).

Fig. 2.8: A schematic representation of the pGL3-Basic reporter vector containing the Luciferase gene (LUC). The ampicillin resistance gene enables selection on the basis that only colonies containing the transformed plasmid are able to grow in the presence of ampicillin.



2.4.2 Transformation

E. coli DH5α™ competent cells (50μL) (Invitrogen, Paisley UK) were thawed on ice. 2-3μL of plasmid DNA (~800ng) was added, mixed gently by swirling with a pipette tip and then incubated on ice for 20 minutes. The mix was 'heat shocked' at 42°C for two minutes, followed by two minutes in an ice bath. The competent cells were then added to 37°C pre-warmed L-Broth (1ml) and incubated with occasional shaking for one hour. The transformation mix (1ml) was then plated using a disposable spreader onto Ampicillin selection agar plates (10ml L-Agar and 100ng/ml Ampicillin) and incubated at 37°C overnight (pGL3-Basic transformation confers Ampicillin resistance on *E. coli* DH5α™ competent cells). A single colony was picked from a freshly streaked selective plate and inoculated in a starter culture of 5ml L-Broth medium containing 100ng/ml Ampicillin. The starter culture was incubated for approximately 8 hrs at 37°C with vigorous shaking (250rpm). The bacterial cells were harvested by centrifugation at 6000G for 15 mins at

4°C. DNA was extracted from 4 mls of bacterial culture using the QIAprep spin miniprep kit (Qiagen Crawley, UK) according to manufacturers instructions (section 2.4.3).

2.4.3 Miniprep and Maxiprep of Plasmid DNA

Miniprep system

For sequencing applications and other small scale experiments, the QIAprep spin miniprep kit (Qiagen Crawley, UK) was used. The miniprep procedure involves the alkaline lysis of bacterial cells followed by high-salt binding to a column. Subsequent washing releases the plasmid DNA. 4ml of bacterial culture was centrifuged at 6000g for 15 min at 4°C. The L-broth was aspirated and the pellet re-suspended in 250μL of buffer P1. 250μL of buffer P2 (alkaline lysis buffer) was then added and the contents transferred to an eppendorf tube. The lysis reaction was left for 5 min before the addition of 350μL neutralising buffer N3. The contents of the tube were gently mixed by inverting 6 times and the tube centrifuged at 10000g for 10 min. The supernatant from this step was then applied to the QIAprep spin column and centrifuged for 30s at 10000g. The column was washed with the addition of 750μL of buffer PE and spun for 2min at 10000g. The column was removed, placed in a fresh Eppendorf and 50μL of distilled water added. After one minute of incubation the tube was centrifuged for 1 min at 18000g in order to elute the plasmid. Concentrations of plasmid were calculated using a spectrophotometer.

The Gene Elute HP maxiprep kit (Sigma-Aldrich, Poole UK)

In order to generate high quantities of plasmid DNA for transfection experiments, a maxiprep kit was used. This system uses similar alkaline lysis of bacterial cells, except in a larger scale. 150ml of overnight bacterial culture was centrifuged at 5000g for 10min at 4°C. The supernatant was carefully discarded and 12ml of resuspension buffer added. 12ml of Lysis solution was then added and mixed by careful inversion of the tube. After 5min, 12ml of neutralisation solution was added along with 9ml of binding solution and the solution thoroughly mixed. The contents of the tube were then added to a filter syringe and left for 5min. Meanwhile, a binding column was prepared in a 50ml tube by the addition of 12ml column preparation solution, and centrifuged at 3000g for 2min. After 5min the filter syringe was emptied (using the provided plunger) onto the binding column. The filtered solution was then centrifuged at 3000g for 2min and the flow through discarded. 12ml of wash solution was added to the column and spun for 5min at 3000g. The washed column

was then transferred to a fresh 50ml tube, 3ml of elution buffer was added, and centrifuged at 3000g for 5min.

The 3ml of eluted plasmid was then ethanol precipitated as follows: 300μL Sodium Acetate (3M, pH 5.6) and 6.6ml of 100% ice cold Ethanol was added to the 3ml plasmid preparation, and left for 24hrs at -20°C. After this time, the tube was centrifuged (10000g) for 30 minutes at 4°C and the supernatant removed. The pellet was then washed with 200μL of 70% Ethanol and allowed to air dry. The pellet was then re-suspended in 500μL of distilled water.

2.4.4 Site directed mutagenesis

Site directed mutagenesis (SDM) was used to introduce point mutations, and performed using the Quikchange-SDM kit supplied by Stratagene (California, USA) as follows. PCR was performed in a total volume of 50μL containing two complimentary oligonucleotides (125ng) of between 25-35 base pairs in length containing the intended point mutation that wished to be introduced. Also included was 1× reaction buffer, 50ng plasmid DNA, 0.25mM dNTP mix and 1μL PfuTurbo™ DNA polymerase (2.5 U/μL). The reaction was overlaid with 50μL paraffin oil. The PCR conditions were as advised by the Stratagene kit with an annealing temperature of 50°C for 1 minute and extension of 68°C for 7 minutes (15 cycles). 1μL of *Dpn I* was added to the reaction mixture (one hour at 37°C) in order to remove the original methylated template plasmid DNA (*Dpn I* only recognises and cuts methylated DNA). *E. coli* DH5α™ competent cells were then transformed as described in section 2.5.1 in order to generate the newly mutagenised plasmid. However, in order to generate large quantities of plasmid, single transformed colonies were picked and grown in 150ml of L-Broth medium containing 100ng/ml Ampicillin at 37°C for 8 hours. Plasmid was isolated using the Gene Elute HP maxiprep kit (Sigma-Aldrich, Poole UK) according to manufacturers instructions and ethanol precipitated (section 2.4.3).

2.5 Sequencing

Sequencing was based on the chain terminator sequencing method (Sanger and Coulson, 1975) utilising BigDye Terminator v3.1 Cycle Sequencing chemistry. The ddNTPs are labelled with four dichlororthodamine fluorescent dyes (dR110, dR6G, dTAMRA and dROX) modified to produce 2-3 times brighter fluorescence and an increase in overall sensitivity. The four dyes (one for each base) allow the sequencing instrument to

recognise the distinct label colours and therefore give the sequence information in a single reaction mixture.

Template DNA

Purified pGL3-Basic plasmid DNA was used as template DNA. Before sequencing, plasmid was purified using either the QIAprep spin miniprep kit (Qiagen, Crawley UK) or Gene Elute HP maxiprep kit (Sigma-Aldrich, Poole UK) according to the manufacturers recommended instructions (section 2.5.3). Oligonucleotides relevant to the region of DNA to be sequenced were designed using Oligonucleotide 3 software as described in section 2.2.3.1 (Table 2.5).

Table 2.5: Oligonucleotides used for sequencing reactions. The pGL3-Basic oligonucleotides were used to check that the fragment of interest had successfully ligated into the plasmid vector. Because sequencing is limited to ~500 base pairs, several sets of internal oligonucleotides were designed in order to sequence the entire *PLA2G7* promoter region

Sequencing Target	Oligonucleotides
pGL3-Basic 5' polyclonal site	5'-CTAGCAAAATAGGCTGTCCC-3'
pGL3-Basic 3' polyclonal site	5'-CTTTATGTTTTTGGCGTCTTCC-3'
PLA2G7 Promoter (1) Forward	5'-CCCAAGGTCACACCACTCAT-3'
PLA2G7 Promoter (1) Reverse	5'-CTCCAAACCCGACTGCTTCT-3
PLA2G7 Promoter (2) Forward	5-ATTTGCATTTCGTGCTCACA-3
PLA2G7 Promoter (2) Reverse	5'-AACTGAAACGCCTTCCTTCA-3'

Sequencing reaction

The sequencing reaction was performed using 2μL of 5× Buffer, 2μL BigDye dNTP mix, 30-100ng of PCR/plasmid template DNA, 1.6pmol of either forward or reverse oligonucleotide made up to 10μL with dH_2O. The reaction mixture was overlaid with 10μL of paraffin oil and placed on a PCR block. The conditions of the cycle sequencing reaction were: 96°C for 15 seconds × 1 cycle, followed by [96°C for 45 seconds, 50°C for 45 seconds, 60°C for 3 minutes] × 25 cycles.

Ethanol precipitation

Following the sequencing reaction, samples were ethanol precipitated to remove excess dye terminators. The 10μL sequencing reaction product was removed from under the oil and transferred to a fresh eppendorf tube, avoiding transfer of any paraffin oil. 7.5M

ammonium acetate (3.5μL) and 34μL of 95% ethanol were added to the tube, vortexed briefly, and left at room temperature for 20 min. The tube was spun at 12000g for 30 min and ethanol removed with a pipette leaving the intact pellet. The pellet was washed with 250μL of freshly made 70% ethanol, and the tube span at 12000g for 10 min. Again the ethanol was removed, carefully avoiding disturbance of the pellet. The pellet was left to air dry for 20 min in a darkened container.

Gel electrophoresis

5% acrylamide-urea gels were used for sequence determination. 20ml of Sequagel XR rapid sequencing solution (containing 19:1 bisacrylamide:acrylamide and urea) and 5ml of Sequagel Buffer reagent (Containing TBE and Temed) were mixed in a beaker briefly. The gel was polymerised by the addition of 250μL of APS and poured between two pre-cleaned (with dH_2O) ABI sequencer glass plates. Once set, the plates were cleaned thoroughly and any excess polymerised gel removed. The gel was then placed into an ABI 377 Sequencing machine and pre-warmed by running at 65W (1600V, 80A) in 1x TBE buffer for 30 min. The samples were re-suspended in 6μL of sequencing loading dye, denatured at 95°C for 5 min, and placed into ice water to prevent re-annealing. 2.5μL of the sample was then loaded onto the saw-tooth wells of the gel, and the gel run in 1x TBE buffer at 1600V for 7 hours using ABI 377XL collection software. Lane and base calling was achieved using ABI 377XL sequence analysis software v3.3 (Applied biosystems, California USA).

2.6 Two Step Real-Time PCR

2.6.1 Step 1: RNA extraction and cDNA synthesis

Messenger RNA (mRNA) isolation

All materials were handled with gloves, pipettes autoclaved prior to use for RNA work, and bench-space cleaned meticulously with 70% ethanol. Isolation was performed using the RNeasy kit (Qiagen, Crawley, UK) spin protocol. This entails the use of a high-salt buffer system which allows up to 100μg of RNA longer than 200bp to bind to a silica-gel based membrane with a RNeasy mini column. Initially, a maximum of 350μl of lysis buffer (RLT buffer) together with β-mercaptoethanol (β-ME) were added to stabilise the sample. Following this, 350μl of 70% ethanol was added and the suspension mixed with a pipette. The sample was then added to a spin column and centrifuged at 100g for 15s. The flow-through was discarded and 350μl of buffer RW1 added to the column, and the sample centrifuged for 100g for fifteen seconds. 80μl (70μl buffer) DNase I solution (Qiagen, Crawley UK) was added directly to the silica gel membrane of the spin column for 20min followed by a second RW1 wash. The sample was then washed twice with 500μl of buffer RPE. Finally, the RNA was eluted with 30μl of RNase free water added directly to the silica membrane with subsequent centrifugation for one minute at 100g into a fresh eppendorf. The RNA was stored at -80°C.

Complementary DNA (cDNA) synthesis

The concentration of mRNA was determined by measuring the absorption at 260nm in a spectrophotometer. RNA (~1.5μg or 10μL if the concentration was lower) was added to 2μl random oligonucleotides (pd[N]$_6$, 100ng/μL), 1μL dNTPs (10mM) and the reaction volume made up to 13μl with RNase free water. The sample was heated to 65°C for five minutes and then chilled on ice briefly. 4μl of 1st strand buffer (Invitrogen Ltd, Paisley UK) and 2μl 0.1M DTT was mixed in gently by pipette, followed by incubation at 42°C for two minutes. After this stage, 1μl Superscript II reverse transcriptase (Invitrogen Ltd, Paisley UK) was added, the sample mixed and incubated at 42°C for one hour. The incubation temperature was increased to 70°C for 15 minutes with the subsequent addition of 1μl RNaseH (Invitrogen Ltd, Paisley UK) and incubation for 20 minutes at 37°C. The resulting DNA samples were stored at -20°C.

2.6.2 Step 2: Real Time-PCR (RT-PCR)

Oligonucleotide design and principle

Forward and reverse oligonucleotides and probes were ordered and supplied through the Applied Biosystems 'assay on demand' service (www.allgenes.com) (Table 2.6). Taqman® RT-PCR uses the same chemistry as allelic discrimination (genotyping), with the exception of a single probe labelled at the 5' with 6-carboxyfluorescein (FAM) and a non-fluorescent quencher at the 3' end. Due to intronic splicing of messenger RNA (mRNA), the oligonucleotides were designed within translated exons (spanning an intron). Standard PCR conditions as advised by Applied Biosystems were programmed into the ABI 7900HT Taqman® machine (50°C for two minutes, 95°C for ten minutes, followed by 40 cycles of 95°C for fifteen seconds and 60°C for one minute). In contrast to allelic discrimination where the absolute fluorescence of each allele specific probe was measured after PCR had taken place, relative quantification was achieved by the 7900HT machine taking constant readings of FAM dye fluorescence after each PCR cycle.

Table 2.6: Applied Biosystems Assay design service was used to develop the relevant gene expression assays. All assays were previously tested to ensure accuracy.

Gene	Assay ID	Probe Sequence
PLA2G7	Hs00173726_m1	FAM-ATGAAATCATCAGCATGGGTCAACA-NFQ
Ubiquitin-C	Hs00824723_m1	FAM-GTGATCGTCACTTGACAATGCAGAT-NFQ
GAPDH	Hs99999905_m1	FAM-TTGGGCGCCTGGTCACCAGGGCTGC-NFQ
β-actin	Hs99999903_m1	FAM-TCGCCTTTGCCGATCCGCCGCCGCCCGT-NFQ

Selection of endogenous controls

In order to normalise for differences in total amounts of RNA or DNA that may be present in each of the samples, endogenous controls of each sample and replicate were included. Suitable endogenous control genes are those where expression is relatively consistent in that particular cell line (essentially 'housekeeping' genes), especially when cells are being treated to different conditions that may alter the expression of a target gene, in this case, *PLA2G7*. In order to determine the most suitable control gene for these experiments, the REST-384 algorithm was used (Vandesompele et al., 2002). REST-384 is able to compare the relative stabilities of up to 12 housekeeping genes and identify the most stably

expressed control gene in a given set of tissues or samples (by the calculation of a normalisation factor). The relative expression of three standard control genes; Glyceraldehydephosphate dehydrogenase (GAPDH), β-actin, and Ubiquitin-C (UBC) were compared in 12 different cDNA samples in order to identify the most suitable endogenous control gene.

Relative Quantification

Table 2.7 lists the standard assay conditions for each individual well of a 384 plate (plates were sealed and centrifuged as described in section 2.2.6). The reverse transcribed cDNA was used as a template for RT-PCR with each cDNA sample run in triplicate for both the endogenous control and target gene. Relative quantification of target *(PLA2G7)* mRNA was performed with SDS 2.1 software, after amplification and detection with the ABI Prism 7900 Sequence Detection System. The Cycle threshold (Ct), at which a significant increase in PCR cycle threshold was detected, was automatically set at the beginning of the logarithmic phase of each PCR amplification, in each well on the plate. The Ct value was defined as the fractional PCR cycle number at which the fluorescence emitted from a particular well rose above the threshold. The difference in Ct values for an endogenous reference, and *PLA2G7* was used as a basis for the relative expression analysis. Comparisons were then made between changes in expression of *PLA2G7* following macrophage exposure to Simvastatin. Macrophages that were not treated were set as the reference point. Each sample was run in triplicate (for both *PLA2G7* and the endogenous control) and the mean Ct value used for analysis. The lower the Ct value, the higher the amount of mRNA in the sample tested. The difference for each sample relative to endogenous control was calculated (following the manufacturers instructions) as follows:-

$$\Delta Ct = (Ct\ PLA2G7) - (Ct\ Endogenous\ control)$$

A comparative Ct (ΔΔCt) was obtained for the treated sample relative to the reference sample using the formula:-

$$\Delta\Delta Ct = (\Delta Ct\ Treated) - (\Delta Ct\ Reference)$$

The amount of mRNA, normalized to an endogenous reference and relative to a calibrator (assuming 100% efficiency) is given by:-

$$2^{-\Delta\Delta Ct} \text{ or } 2^{-(\Delta Ct\ Stimulated)-(\Delta Ct\ Reference)}$$

Table 2.7: Assay reagents for a RT-PCR. 4μL was carefully pipetted into each well of the 384 well plate using a P10 pipette after 1μL (P2 pipette) of cDNA had been added and centrifuged to the bottom of the well.

Reagents for a 20x Gene expression assay mix	**Volume in μL per well**
Taqman Absolute QPCR Rox mix	2.5μL
Assay	0.25μL
Sigma dH_2O	1.25μL
cDNA	1μL

2.7 Cell culture studies

Cell culture was performed in the tissue culture suite of the Rayne Building at UCL, which is a dedicated, protected environment with air filtration and two door locks before entry. Gloves, cell culture dedicated laboratory coats and overshoes were worn at all times. Sterility was maintained by handling all open culture media, culture-ware and liquids within a class II microbiological safety cabinet with unidirectional laminar flow (Envair UK Ltd). Surfaces were cleaned with 1% virkon and 70% ethanol solutions. Materials for use within the laminar flow hoods were pre-sprayed with 70% ethanol. Cell contaminants, such as media used for growth, were immersed in 1% Virkon and stored for at least twenty-four hours before disposal. Galaxy R CO_2 incubators (Wolf Laboratories) humidified and set to 5%CO_2, 37°C were used for cell incubation.

2.7.1 Monocyte culturing

Monocyte extraction was adapted from previously described techniques (Wan et al., 1993; Dentan et al., 1996). After centrifugation of 40mls of EDTA collected venous blood (200g for ten minutes), 100µl of plasma from each subject was aliquoted into wells of a sterile 96 well Nunc cell culture plate (Roskilde, Denmark). Care was taken to carefully label the wells for individual subjects. The plasma was left coating the wells for forty-five minutes at 37°C. Pre-incubating the plates with plasma in this way increased the success of monocyte adhesion. The wells were then washed twice with 100µl of PBS.

The remaining blood was transferred to two 50ml sterile centrifuge tubes with 20mls Macrophage serum free medium (MSFM, supplemented with penicillin at 100units/ml and streptomycin at 100µg/ml), pre-warmed by incubation in a water bath at 37°C. The samples were mixed gently by inversion. 8× four ml of Ficoll-paque (warmed to room temperature) was dispensed to eight fresh 30ml tubes. The blood-MSFM mix was then gently layered onto the surface of the Ficoll-paque. The samples were centrifuged at 200g for twenty minutes at room temperature.

After centrifugation, the monocyte layer is visible between the Ficoll-Plaque and MSFM-plasma layer. The monocytes were then recovered with a pasteur pipette and transferred to a 50ml tube. An equal volume of MSFM was added, and the sample mixed gently and

centrifuged at 200g for five minutes. The supernatant was aspirated, carefully ensuring that the pellet was left intact. A repeat wash was then performed.

The pellet was then re-suspended in 5mls of MSFM including 10% foetal bovine serum (FBS), and the number of monocytes counted. Using this technique the recovery of monocytes is around $5x10^6$ from 4mls of blood. Monocytes were plated at $1x10^6$ cells/well in a sterile 96 well Nunc cell culture plate (Roskilde, Denmark). After 48 hrs 50-70% of the monocytes had adhered and the medium was replaced with 200μL of fresh MSFM. The cells were left for 7 days in order to allow cell differentiation. After this period, the medium was replaced (200μL), removing all the non-adhering cells, and predominantly leaving differentiated macrophages. After a further 48hrs the medium was replaced with MSFM containing 5% FBS. After a total of 11 days in culture the cells were ready for experimentation.

2.7.2 Huh-7 cell culturing

Huh-7, obtained from the European Collection of Cell Cultures (ECACC, Salisbury UK), were routinely cultured in Dulbecco's minimal essential medium (D-MEM) supplemented with 10% foetal bovine serum (FBS) in 75ml flat tissue culture flasks. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Once 90% confluency was reached (determined using an inverted microscope), the cells were 'split' 1:3-1:6 using trypsin/EDTA (Invitrogen, Paisley UK): cells were aspirated of their medium, and washed with 10 ml 1×PBS in order to remove any trypsin inhibitors. 5ml of trypsin (0.05%)/EDTA (0.02%) solution was added and the cells were incubated at 37°C for 4 min. After this time, the flask was tapped to release the adherent cells and 10ml of fresh D-MEM was added. The 15ml was then centrifuged in a sterile 15ml Falcon tube for 5 min at 120g. The medium was aspirated and the pellet reconstituted into 5ml of medium. 20μL of the cell suspension was used to count cell number, and placed between a cover slip and a 1mm deep Haemocytometer. Cells were counted in 4 squares and averaged; the average cell count was equal to $\times10^4$ cells/ml. Cells were seeded to a final concentration of 2 $\times10^5$ cells/ml.

In order to preserve cells, reduce the risk of contamination, and minimise effects of genetic drift, Huh-7 cells were frozen for long term storage. A cryo-protective agent such as dimethylsulphoxide (DMSO) is used in conjunction with complete medium for preserving

cells at -80°C. DMSO acts to reduce the freezing point and allows a slower cooling rate, thus reducing the risk of the formation of ice crystals and cell damage. Cells in log-phase were trypsinised and the cell pellet was re-suspended in 50% complete medium (including 10% FBS), and 50 % freezing medium (Invitrogen, Paisley UK). Cells were mixed thoroughly and placed onto wet ice. Cells were counted using a Haemocytometer as described previously. Aliquots (1ml) of cell suspension (10^6 to 10^7 cells) in a 2ml cryovial were placed at -80°C overnight, then transferred to liquid nitrogen for long term storage. For resuscitation, cells were taken from liquid Nitrogen storage and placed into a water bath pre-set to 37°C. 8 ml of pre-warmed D-MEM medium was placed into a 25ml flat bottomed culture flask. 1ml of fresh medium was added to the cryovial drop by drop then the contents were poured gently into the 25ml flask. After 48 hours the cells were split into a larger 75ml flask by the method described previously in this section.

2.7.3 HL-60 and THP-1 cell culturing

HL-60 and THP-1 suspension cell lines were obtained from the ECACC. Both cell lines are immortalised and monocytic in origin, and are easily differentiated into macrophages by the addition of Phorbol 12-myristate 13-acetate (PMA/TPA). THP-1 and HL-60 cells were cultured in suspension in RPMI-1640 medium (Sigma-Aldrich, Poole UK) supplemented with 10% foetal bovine serum and 2mM L-Glutamine in upright 75ml tissue culture flasks. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Both cell lines were stored in freezing medium as described in section 2.7.2. However, DMSO causes premature differentiation of both cell lines, therefore freezing medium that lacked DMSO as a freezing agent was used (Invitrogen, Paisley UK). For resuscitation, cells were taken from liquid Nitrogen storage and placed into a water bath pre-set to 37°C. 6 ml of pre-warmed RPMI medium was placed in a 25ml tissue culture flask. 1ml of fresh medium was added to the cryovial drop by drop then the contents were poured gently into the 25ml flask. After 48 hrs of incubation, the cells were spun at 120g for 5 min in a 15ml tube and the medium gently removed (to remove any freezing contaminants and cell debris). Cells were then re-suspended in 10ml of medium and incubated in the same medium for 7-9 days to allow log-phase growth. In order to count cell concentration, the cells were spun down at 120 g for 5 min and re-suspended into 20ml of medium. 10μL of the cell suspension was diluted in 90μL of medium, then placed on a haemocytometer. The average count of 4 large squares were taken as the concentration $\times 10^4$ cells/ml (after correction for the 1 in ten dilution). Cell suspensions were maintained between 2 and 9

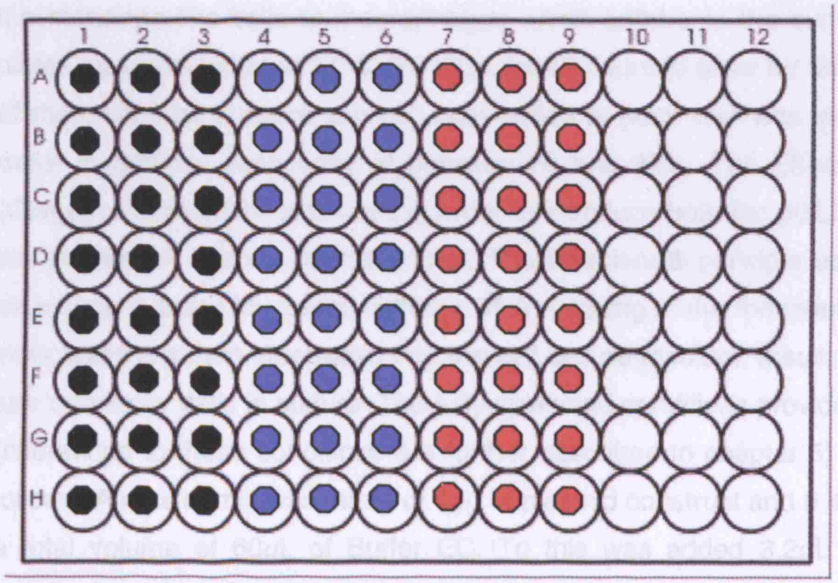
$\times 10^5$ cells/ml, since THP-1 and HL-60 cells tend to differentiate at concentrations higher than 1 $\times 10^6$ cells/ml.

2.8 Functional studies

2.8.1 Macrophage exposure to Simvastatin

Differentiated macrophages from individual subjects were exposed to Simvastatic acid in order to determine whether the expression of *PLA2G7* was altered. The active form of Simvastatin was extracted from a Zocor® tablet (Merck, USA) and kindly donated by Elisabeth Teissier (Pasteur Institute- Lille, France) at a concentration of 43.4mM diluted in 1ml of PBS (pH7.4). Each column of the 96 well plate was treated as a single experiment, with the lysed cells of each column being pooled together in order to extract mRNA (Fig. 2.9). 3 columns (3 separate experiments) were designated as controls with no addition of Simvastatic acid, 3 columns were exposed to 10μM Simvastatic acid, and 3 columns exposed to 25μM Simvastatic acid.

Fig. 2.9: Layout of the Simvastatin treatment for one patient. Each coloured well reflects a different concentration of statin applied. In each column, cell lysate was pooled, therefore each exposure was effectively triplicated.



24 hours before treatment, the medium (MSFM containing 10% FBS) was removed and the cells washed with 200μL of 1×PBS solution. The PBS was carefully removed and 200μL of MSFM supplemented with 5% FBS was added to each well of the 96 well plate. On the day of treatment 3 volumes of medium were made up accordingly:

Control: 9.3μL of 1×PBS (pH7.4) diluted into 8ml of MSFM (5%FBS)

10μM: 1.86μL of Simvastatic acid and 7.44μL 1×PBS diluted in 8ml MSFM

25μM: 4.65μL of Simvastatic acid and 4.65μL of 1×PBS diluted in 8ml MSFM

The medium was removed carefully and the cells washed in 200μL of 1×PBS. The PBS was then removed and 200μL of the treated/control medium was added to each well using an Eppendorf multi-channel pipette. Cells were incubated in 5% CO_2 at 37°C for 48 hours before mRNA extraction. In order to extract mRNA from multiple wells, each well was washed twice with 200μL of 1×PBS and 45μL of the RLT buffer was added using a multi-dispensing pipette. The lysed buffer was then pooled from each column and mRNA extracted according to section 2.6.1.

2.8.2 Assaying of promoter activity

2.8.2.1 Transfection of HL-60 (THP-1) cells with promoter constructs

Promoter constructs were transfected into THP-1 and HL-60 cell lines as follows. 5^{th} to 10^{th} passage cells were split into 6 well plates, with each well containing 1×10^6 cells in 2ml of medium. RPMI medium was supplemented with 20nM PMA leading to the differentiation of the monocyte-like cells to macrophages which adhere to the surface of the well. 6 well plates were incubated at 37°C; 5% CO_2, for 48 hours to allow for differentiation/ adherence of the cells. The concentration of cells added to each well was previously determined in order to attain a confluency of between 70 and 80%. The Effectene® Transfection Kit (Qiagen, Crawley UK) was used in order to introduce both the pGL3-Basic constructs, and the Renilla-TK internal control vector. The Effectene® principle uses lipid based micelle structures that are incubated with the DNA resulting in the formation of complexes. These complexes are then introduced into the cell culture medium, resulting in membrane fusion and uptake by cells in culture. The fully optimised conditions provided by our collaborators (alterations to these conditions are further described in chapter 5) for each well were as follows: A master mix was made of 1μg of plasmid construct and 0.4μg pRL-TK (Renilla) in a total volume of 60μL of Buffer EC. To this was added 3.2μL of Enhancer solution, vortexed and incubated for 5 minutes at room temperature. After this period, 10μL of Effectene reagent was added, mixed by pipetting up and down 5 times, and incubated for

15 minutes. While the complex was incubating, the cells were washed twice with normal growth media, and 0.5ml of RPMI containing 10% FBS was pipetted into each well. After the 15 minute incubation, 0.35 ml of RPMI was added to the complex, mixed by pipetting up and down twice and immediately added drop-wise to the cells. The cells were incubated at 37°C; 5% CO_2, for 24 hours and were then washed with RPMI medium, with the addition of 2ml of normal growth medium. After 48 hours the cells were ready for the transfection to be tested. In order to transfect 12 well plates appropriately, the relative surface area of the 6 well dish was compared to that of a 12 well. This ratio provided a scaling down fraction for all the reagents used. In addition, the manufacturer's protocols provided optimised volumes for 12 well dish transfections, and are therefore not shown in this chapter.

2.8.2.2 Huh-7 transfection

Huh-7 cells were trypsinised and counted as described in section 2.7.2. Cells were plated out at a density of 4×10^4 cells (in 100μL of D-MEM) per well of a 96 well plate, and incubated at 37°C; 5% CO_2, for 24 hours to enable proper stabilisation of the cells. For this particular cell line Lipofectamine 2000™ was used for transfections. This product acts in a similar way to the Effectene® reagent with a lipid based delivery system, but unfortunately requires low serum medium. In order to transfect 8 individual wells of a 96-well dish for each construct, a master protocol was devised as follows: A master mix of 2988μL of OptiMEM (Serum free medium, Invitrogen UK) and 12μL of pRL-TK plasmid (1ng/μL) was made up and left for 5 minutes on the bench. For each construct of interest (see chapter 5 for more details) 245μL of this master mix was added to 5μL of plasmid construct (400ng/μL) and incubated for 5 minutes at room temperature. In another eppendorf, a lipid mix was made up of 60μL of Lipofectamine 2000™ reagent and 2940μL of OptiMEM. 250μL of the lipid mix was then gently added to the master mix and incubated at room temperature for 20 minutes. After this time, cells were aspirated and washed twice with 1× PBS solution, and 50μL of the combined mix was added to each well. After 6 hours incubation at 37°C; 5% CO_2, the cells were aspirated and the medium replaced with 100μL of D-MEM medium. After a further 48 hours of incubation, the transfections were ready for testing.

2.8.2.3 Reporter gene assay

Promoter activity was assayed using a dual luciferase assay. The activities of Firefly and Renilla luciferases were measured sequentially from a single sample. The firefly luciferase reporter was measured first by adding Luciferase Assay Reagent II (LAR II) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction was initiated simultaneously by adding 'Stop and Glo®' reagent to the same tube. The Stop and Glo® reagent also produces a 'glow-type' signal from the Renilla luciferase, which decays slowly over the course of the measurement.

Cells were washed with PBS and 50μL of passive lysis buffer was added to each well for a 6 well plate or 20μL for a 96 well plate. The plate was then incubated at room temperature for 1 hour. The lysed sample (10μL) was used to measure activity in a 96 well plate. Firefly and Renilla luciferase activities were measured in a PerSeptive Biosystems CytoFluor Series 4000 fluorescence multi-well plate reader using a single-injector TD-20/20 Luminometer operating in the 'DLR' mode. TD-20/20 Luminometer software allowed for direct capture of the data stream over the 10-second integration period of each luciferase reaction. Using this system, both luciferase reporter assays were quantified within 25 seconds. Promoter activity was taken as the ratio of the firefly luminescence to Renilla luminescence, thus adjusting for transfection efficiency. Chapter 5 further describes the positive and negative controls used for each experiment.

CHAPTER 3

THE DETECTION OF COMMON VARIANTS IN THE *PLA2G7* PROMOTER REGION

3.1 Introduction

3.1.1 Genetic variation in the Human genome

The sequence of the human genome is not identical between individuals, and variations frequently appear resulting from a permanent change in DNA sequence. If a sequence change is rare and alters the function of the gene (often having a detrimental effect) they are usually described as mutations. By contrast, variations in the human genome with population frequencies of greater than 1% are termed polymorphisms (Barnes and Gray, 2003). Much of the common variation (or polymorphisms) present in the human genome is the result of a changes in single nucleotides, and these have been termed; Single Nucleotide Polymorphisms (SNPs). Within the sequenced human genome there are thought to be several million of these SNPs (a best estimate of 3-10 million)(Lisker, 2004; Goldstein et al., 2003; Venter et al., 2001).

Many of the identified variants or SNPs present in human DNA are 'silent', others may affect either the structure of the protein translated (for instance, the ability of an active site of an enzyme to hydrolyse specific substrates) or the quantity of protein expressed. Sequence changes that have this effect can be described as 'functional gene variants' and may have important biological consequences (Barnes and Gray, 2003). Functional gene variants may result in the substitution of one base pair for another, resulting in the change of a codon. This may lead either to a missense alteration, in which one amino acid replaces another amino acid in a protein altering the protein structure, or to a nonsense alteration in which a termination codon appears in the middle of a gene. When a nonsense codon appears there is no transfer RNA molecule to recognise the codon, therefore protein synthesis terminates and a truncated protein is produced. Variation within RNA-splicing sites may cause mis-splicing in the intron or exon, and this is likely to result in a protein lacking part or all of an exon (or having additional amino acids or in an in-frame 'stop' codon), again altering the structure of the gene product. In addition to changes in the structure of a gene-product, variations may impact upon the *quantity* of protein produced. This occurs if there are changes in the rate or level of gene transcription or messenger RNA stability. Variations in RNA processing could affect the stability of messenger RNA, which in turn may affect the amount of gene product. Variations arising in gene regulatory sequences, such as the promoter region, may alter the rate or level of gene transcription. The function of the promoter region is to set the location and direction of transcription on a

DNA template. RNA polymerase must be recruited to the promoter site, a task mediated by a number of proteins termed transcription factors. Transcription factors bind to sequences within the promoter, or may bind to one another to instruct the RNA polymerase whether or not to transcribe the particular gene. For these reasons, variants present with the gene promoter region are of particular interest.

There is one circumstance in particular where caution is required before ascribing a difference in biological activity to any one variant site. This occurs when two polymorphic sites are in complete or very strong allelic association (linkage disequilibrium-LD). In such a situation one allele of the polymorphism of interest almost always occurs in the presence of a specific allele of the second linked variant. In this circumstance, the gene variant under investigation merely acts as a marker for the presence of the second variant (Crawford and Nickerson, 2005). Thus, the 'effect' of variation in the candidate gene is in fact a reflection of the function of the adjacent functional variant. LD is a population-based concept and is detected in a sample of unrelated subjects, as the occurence together of two alleles of gene variants, more frequently than expected by chance alone. It is usually measured as delta (Δ) or D', and values range from -1 (complete negative association) to +1 (complete positive association)(Devlin and Risch, 1995). Negative values of LD suggest that the common allele of one particular variant is associated with the rare allele of another (or vice versa), and values of 0 represent little or no association. D' differs from Δ, in that D' is standardised on allele frequencies but also takes into account the maximum Δ that could be reached owing to the allele frequencies, and as a consequence is less sensitive to differences in allele frequency (Zondervan and Cardon, 2004; Devlin and Risch, 1995).

3.1.2 Detection of variants

The Single strand conformation polymorphism (SSCP) detection method described in section 2.3 is a robust and powerful method commonly used to identify genetic variation. The mobility of single strands can vary considerably as a result of only a single base pair change in nucleotide sequence. This property led to the development of SSCP techniques (Orita et al., 1989). Under non-denaturing conditions each individual strand (SSCP) or heteroduplex (HD) of DNA will form a secondary structure unique to that particular sequence, and these conformational differences will cause the DNA strands to migrate differentially through an acrylamide gel. SSCP itself can detect a large proportion of the sequence variation in a strand of DNA, typically 150-250 nucleotides in length (Hayashi,

1991; Sheffield et al., 1993), although longer strands of around 400-500 nucleotides have been successfully analysed in our laboratory using a sensitive capillary based system (MegaBACE™ 1000).

3.1.3 Detection of variation in the *PLA2G7* gene

The controversial opposing pro- and anti- atherogenic properties of Lp-PLA2 have been demonstrated both in human and animal models (Hakkinen et al., 1999; Carpenter et al., 2001; Quarck et al., 2001; Noto et al., 2003). From an epidemiological stand point, both activity and mass of Lp-PLA2 in Caucasian populations has been shown to be a consistent risk marker for CHD, independent of traditional risk factors [reviewed in (Sudhir, 2005)], although it is still unclear whether plasma Lp-PLA2 exhibits a causal role, or is a marker of risk (see section 1.3.3). A clearer understanding of the role of Lp-PLA2 in atherosclerosis could be achieved if a functional variant was found that significantly altered expression or activity of the enzyme.

To that end, previous work identified a loss-of-function mutation present in 4% of the Japanese population (V279F), which has also been reported to be associated with an increased risk of CHD (Yamada et al., 1998). This would support the *anti-atherogenic* action of this enzyme. In order to relate the variability within the Lp-PLA2 gene *(PLA2G7)* to atopy and asthma within Caucasian subjects, several other variants have been detected using SSCP (Kruse et al., 2000). Three variants leading to amino acid changes were detected in exon 4 (Arg92His), exon 7 (Ile198Thr), and exon 11 (the Ala379Val change), with the exon 11 variant being found to be potentially functional (Kruse et al., 2000) (Fig 1.13). To date, two studies have found that V379 homozygote individuals are associated with a reduced risk of CHD (Abuzeid et al., 2003; Ninio et al., 2004), although questions remain over the precise effect of the variant on enzyme function (Ninio et al., 2004).

The promoter region of the *PLA2G7* gene has been well characterised with regards to potential transcription factor binding sites that potentially modulate expression of the gene (Cao et al., 1998)(Fig. 3.1). Any interruption to these sites may lead to altered expression of the *PLA2G7* gene, consequently affecting measures of mass in those individuals with the genetic variant. Recent SSCP analysis also identified two novel polymorphisms 5' of the transcription start site (T-403C and C-209G, numbered from the start of transcription)

(Ninio et al., 2004), although no functional data exists concerning their effects on *PLA2G7* expression.

The aim of this aspect of the PhD was to screen the *PLA2G7* promoter region using SSCP and HD analysis, in order to detect any further genetic variation in 48 healthy Caucasian men from a UK population who were participants of the second Northwick Park Heart Study (NPHS II) (Miller et al., 1995). Those samples that exhibited a different SSCP pattern were then sequenced in order to determine the exact nature of the genetic variation. For every identified polymorphism, the frequency and LD was determined with regards to the functional A379V variant. Several database resources are now freely available on the internet (detailed in the methods section of this chapter) that are able to identify potential transcription factor binding sites present in submitted promoter sequences. These resources would be used in order to determine if any of the novel variants interrupted these sites.

3.2 Methods

3.2.1 Study sample

The promoter region of the *PLA2G7* gene was screened in 48 healthy UK Caucasian men from the NPHS II study. Chapter 4 gives a full summary of this study and how individuals were selected.

3.2.2 Polymorphism detection

Overlapping oligonucleotides spanning 1.88kb 5' and 311bp 3' of the previously established *PLA2G7* gene transcription start site (Cao et al., 1998) were designed using Primer 3 software (Rozen and Skaletsky, 2000) and optimised for PCR. Table 3.1 lists the PCR conditions for each set of primers, and Figure 3.1 gives an indication of where the oligonucleotides were placed 5' of the transcription start site. The MegaBACE™ 1000 (Amersham Biosciences, 96 capillaries, DNA sequencing and genotyping system) system was utilised to detect SSCP and HD pattern variation (as described in section 2.3), and Genetic-Profiler v1.5 from Amersham Biosciences was used to analyse any conformational changes. Samples showing variation (all SSCP runs were repeated) were re-amplified using non-labelled oligonucleotides and purified using the QIAprep spin miniprep kit (Qiagen Crawley, UK) according to the protocol listed in section 2.4.3. Samples were then sequenced utilising BigDye Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems, California USA) in accordance with the protocol listed in section 2.5. Forward and reverse strands of DNA were sequenced (using either forward or reverse oligonucleotide in the sequencing reaction) in order to confirm any potential differences.

3.2.3 Allele frequencies

The three potential variants found by SSCP and sequencing analysis were genotyped in 192 randomly selected individuals from the NPHS II study. One of the sequence changes altered a naturally occurring restriction enzyme recognition site, with PCR and digest performed as described in section 2.2.3. Two of the potential variants did not disrupt a natural restriction site, therefore a 'forced' site was developed using either a forward or reverse oligonucleotide complementary to the sequence apart from one single base change. Any PCR amplified strands of DNA would therefore incorporate the altered base, providing a novel restriction site close to the potential variant. Table 3.2 shows the PCR

conditions and digest enzymes used for the three potential variants found. Digestion of PCR products and visualization of MADGE gels was performed as described in section 2.2.3.

3.2.4 Potential transcription factor sites

Two programs were used to predict the location of transcription binding sites within the promoter region of the *PLA2G7* gene. The transcription element search system (TESS) is freely available on the internet (http://www.cbil.upenn.edu/tess/), and collates information from the TRANSFAC, IMD and CBIL-GibbsMat databases. TESS identifies potential transcription binding sites in submitted DNA sequences, and weights them according to sequence homology (Schug and Overton, 1997). TRANSFAC® 7.0 is a similar extensive database of eukaryotic transcription factors, which was also used to search the *PLA2G7* promoter sequence (Matys et al., 2003).

3.2.5 Statistical analysis

Data was entered into excel spreadsheets and analysed using SPSS v12.0.1 (SPSS Inc., Chicago USA) by myself. Deviations from Hardy-Weinberg equilibria were considered using chi-squared tests. Hardy-Weinberg equilibrium gives the expected genotype distribution based on the observed frequency of the rare allele (q) and common allele (p) as $p^2+2pq+q^2$, where p^2 is the predicted frequency for homozygosity of the common allele, q^2 is the predicted frequency for homozygosity of the rare allele and $2pq$, the heterozygotes. These frequencies are expected provided the sample is drawn from a population with random mating and no strong selection. Deviations from the expected frequencies may suggest selection bias or technical problems with the method of genotyping. Allelic association was considered using the statistic delta (Δ) as described previously (Chakravarti et al., 1984). Lewontin's D' was determined from the Haploview 3.2 program developed by the Broad institute, Cambridge USA (http://www.broad.mit.edu/mpg/haploview/). Δ is essentially a correlation coefficient between different alleles (equivalent to Pearson's correlation coefficient) while D' is a scaled down version of D (between 0 and 1) which itself is equivalent to a co-variance between loci (Zondervan and Cardon, 2004; Devlin and Risch, 1995). P-values <0.05 were considered significant.

Fig. 3.1: Schematic of the *PLA2G7* promoter, including the positions of the overlapping forward and reverse oligonucleotides. The previously studied proximal promoter region is also displayed (Cao et al., 1998).

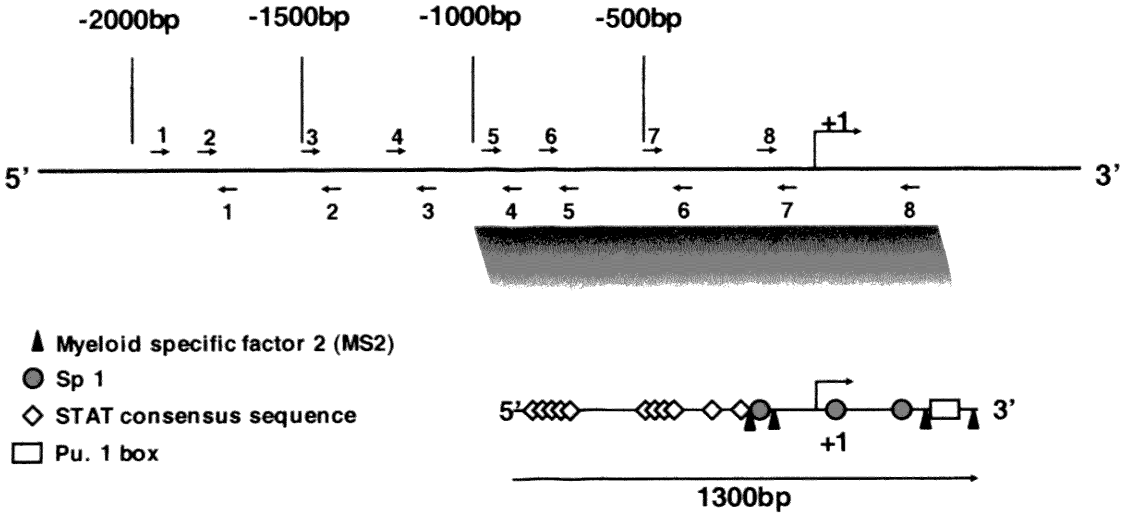


Table 3.1: Overlapping primer pairs used in SSCP analysis of the *PLA2G7* promoter region. Primer set 1 is defined as being the furthest 5' from the transcription start site. The position of the forward oligonucleotide (labelled by FAM) in relation to the start of transcription is set out in brackets.

Primer set	Oligonucleotides	Mg^{2+}	Annealing temperature	Cycles	Fragment length (bp)
1	5' FAM-TTGATTGAAGGGAAGAGGTGAGAC-3'	2mM	59°C	35	259
(-1882bp)	5' HEX-GGCTTCCTGTATTATATCTGGCAAC-3'				
2	5' FAM-TGTCTGTGTTGACTTATCCTCTTGG-3'	3mM	65°C	29	290
(-1711bp)	5' HEX-AGTTGGCATTTGGCAGGTTAGT-3'				
3	5' FAM-TGGGCACTCTTCACTGGGCAT-3'	2.5mM	63°C	35	302
(-1475bp)	5' HEX-GCCCCACAAATATCAATCTAAAAGGA-3'				
4	5' FAM-GACGTTAGAGATGTCTTTGGTAGGG-3'	2.5mM	66°C	35	335
(-1232bp)	5' HEX-GCTTCTCGGTCTCCTCATCCATAAA-3'				
5	5' FAM-TGCATTTCGTGCTCACAAACTGC-3'	3mM	65°C	29	230
(-975bp)	5' HEX-TTAAATCGTGTTTTGGTTTTTGGTG-3'				
6	5' FAM-TAAGCAAAATTTCATCTCCAGTTCTG-3'	2.5mM	67°C	35	414
(-802bp)	5' HEX-TTTTCCGCACTCCGATACACTTTA-3'				
7	5' FAM-AGGGAGAAATGACACCAGCACAG-3'	2.5mM	69°C	35	454
(-492bp)	5' FAM-CTCTCCTCCCCTGGTGATGCT-3'				
8	5' FAM-ATGGTCTTGGGGAGGGTGCT-3'	2mM	63°C	35	478
(-167bp)	5' HEX-AGCGGGTGCGACTCAGCGA-3'				

Table 3.2: A) PCR conditions for the three potential variants found (forced base pairs are in bold). B) Restriction digest condition – PCR products were incubated overnight before MADGE gels were run.

A.

Gene variant	Oligonucleotides	$MgCl_2$	Annealing temperature	Number of cycles	Fragment length
PLA2G7 **C-1700A**	FOR 5'-ACCCATATCAGACACACCTTATAA**G**-3' REV 5'-AGATTTAACTGGGCTTCCTGTATT-3'	2.5mM	62°C	34x	129bp
PLA2G7 **C-1231T**	FOR 5'-ACTTTGTCTTCACCTTTGTCTTA-3' REV 5'-TCCGCCTAATGACATTGTCTT-3'	2.5mM	61°C	34x	149bp
PLA2G7 **G-1230A**	FOR 5'-ACTTTGTCTTCACCTTTGTCTTA-3' REV 5'-ATGCCCTACCAAAGACATCT**G**TAA-3'	2.5mM	57°C	34x	138bp

B.

Gene variant	Enzyme and units (U) per well	Buffer system	Manufacturer of enzyme and buffer system	Incubation temperature	Fragment sizes produced
PLA2G7 **C-1700A**	*Alu I* 3U	NEB buffer 2	New England Biolabs	37°C	129/103/26bp
PLA2G7 **C-1231T**	*Bbs I* 2U	NEB buffer 2	New England Biolabs	37°C	149/112/37bp
PLA2G7 **G-1230A**	*Mae III* 0.3U	MaeIII 2xbuffer	Roche Diagnostics	55°C	138/114/24bp

3.3 Results

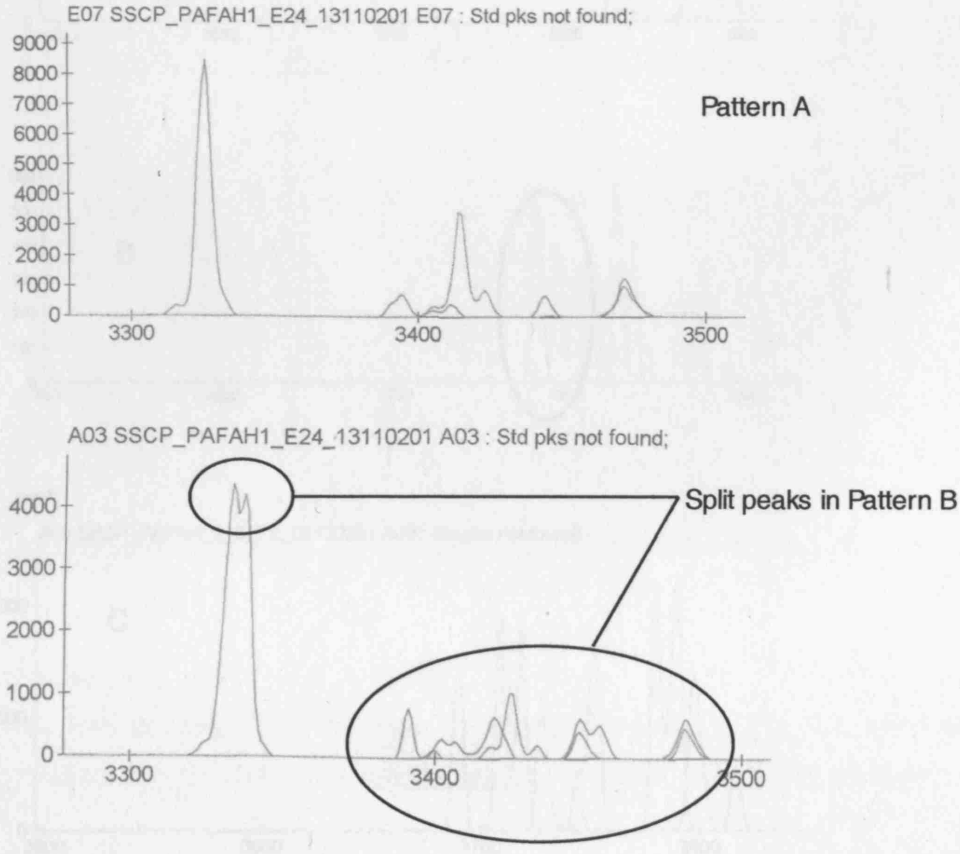
3.3.1 SSCP analysis

Of the eight pairs of overlapping oligo-nucleotides tested, two primer sets produced different SSCP patterns in a proportion of the samples. Heteroduplex analysis showed no variation in all of the primer sets analysed.

Primer set 1

The primer set 1 oligonucleotides amplified a fragment 259bp in length, starting 1880bp 5' of the transcription start site. Of the 48 different samples analysed for this primer pair, one individual's pattern was distinguishable (Pattern B) from a common arrangement of peaks as highlighted in Fig. 3.2 (Pattern A).

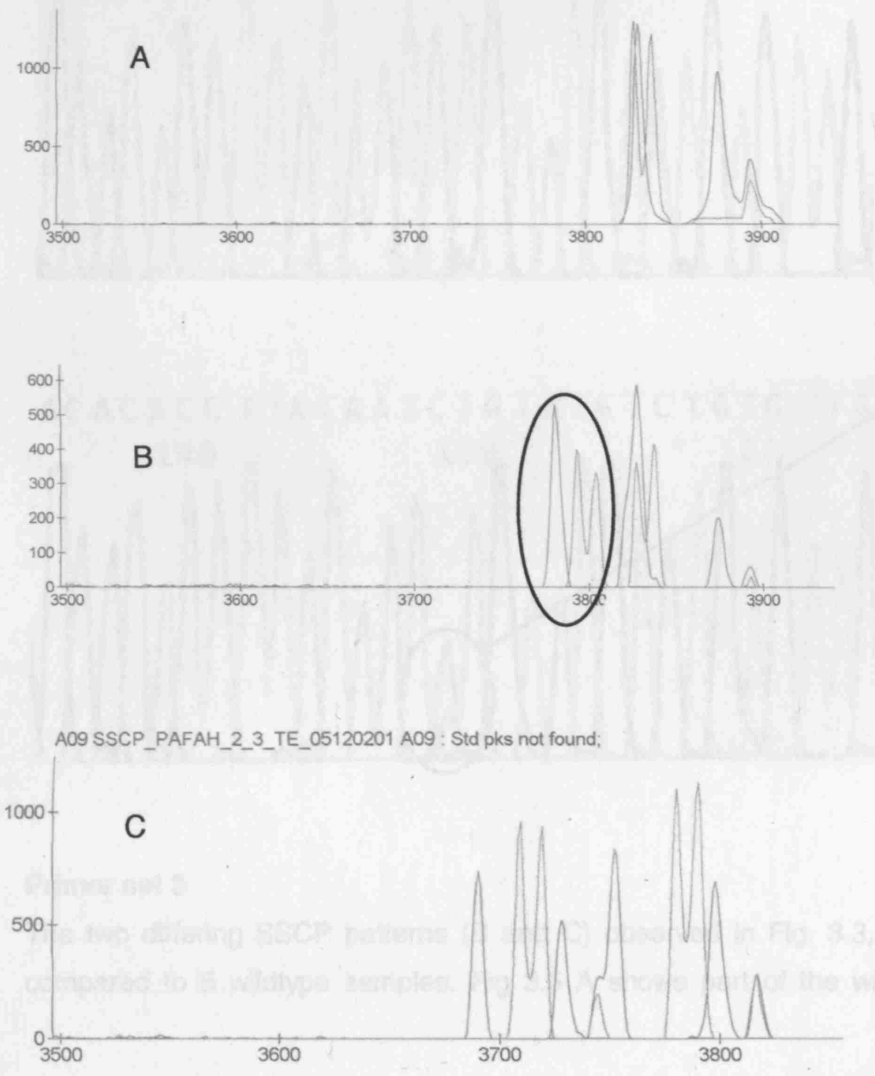
Fig. 3.2: SSCP pattern detected from the MegaBACE 1000 system and analysed using Genetic-Profiler v1.5. Pattern A is the typical peak cluster seen in 47 of the 48 individuals tested. Pattern B exhibits a different pattern as circled, with several 'split peaks' characteristic of a change in DNA conformation



Primer set 3

A 302bp amplified region starting 1475bp 5' of the transcription start site also suggested variation in a proportion of the samples. Three distinct combinations of peaks were observed in 19 of the 48 samples tested. Fig. 3.3 shows the three typical patterns observed; pattern A was the most common and represents the wildtype. Pattern B was present in 18 of the samples and is distinctive from pattern A by the addition of the circled set of peaks. The third pattern (C) was only present in one individual, and differed significantly from either of the previous conformation of peaks.

Fig. 3.3: Three SSCP patterns observed when using Genetic-Profiler v1.5. Pattern A was regarded as the wildtype pattern, while Pattern B was distinguishable by the addition of a second set of peaks circled. Pattern C was only observed in a single sample and is distinct from either of the previous patterns.

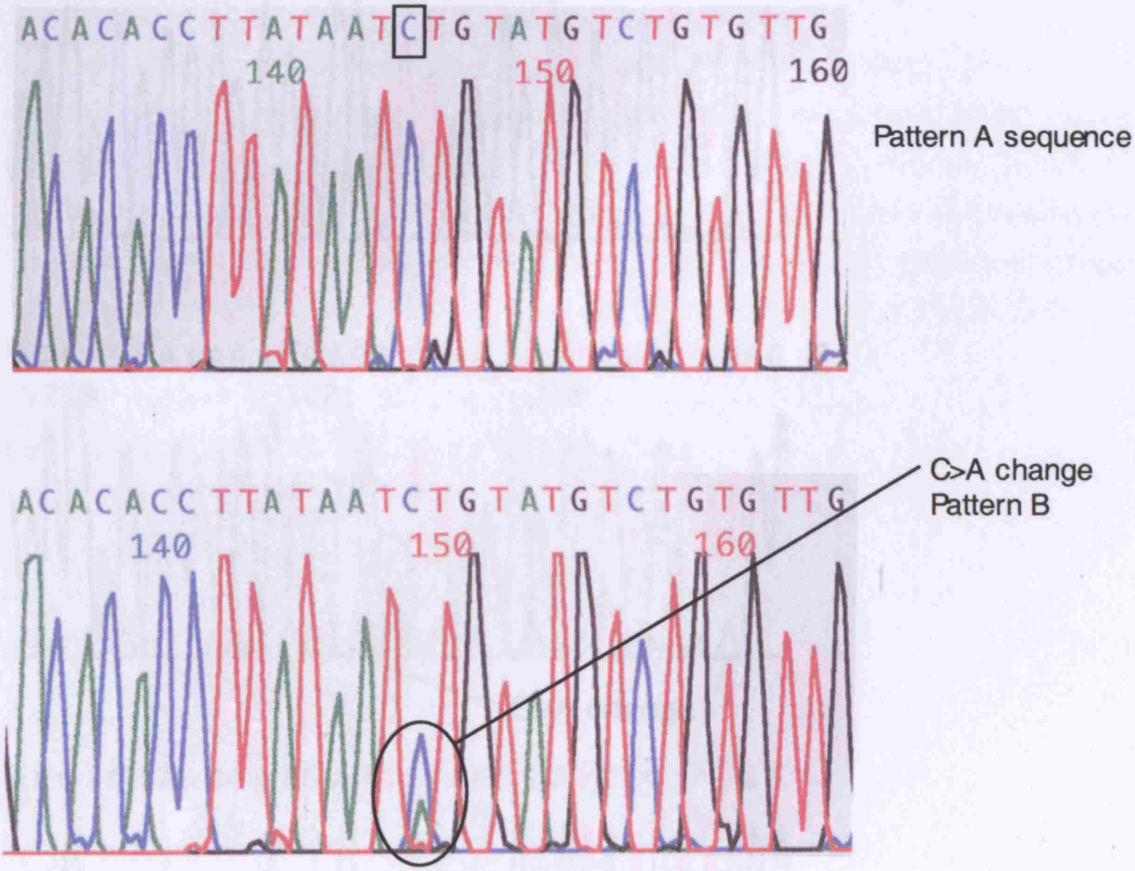


3.3.2 Sequencing of distinctive SSCP patterns

Primer set 1

The change in peak pattern observed for one sample was investigated further by sequencing. Fig. 3.4 shows some of the sequence from the forward strand (the reverse strand confirmed the result but is not shown). The individual with the SSCP pattern B was shown to be heterozygous for a C to A change that appears 1700bp 5' of the transcription start site. Five other wildtype samples (pattern A) were consequently sequenced showing no change to the cytosine. Both pattern A and B sequences were identical apart from this base change.

Fig 3.4: Sequencing results obtained from the forward strand.

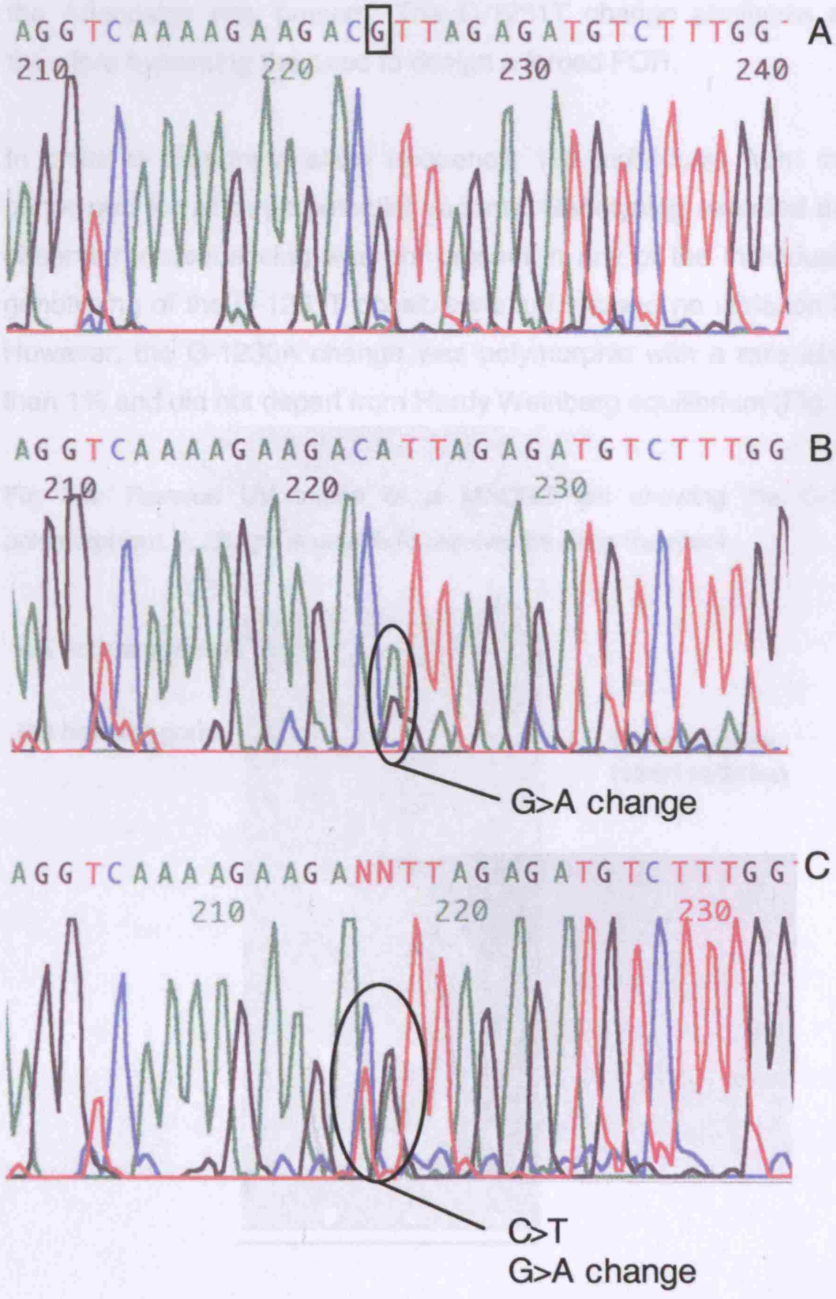


Primer set 3

The two differing SSCP patterns (B and C) observed in Fig. 3.3, were sequenced and compared to 5 wildtype samples. Fig 3.5 A shows part of the wildtype sequence. The

sequence of SSCP pattern B was homologous to the wildtype apart from a G to A change 1230bp 5' of the transcription start site (Fig. 3.5 B). The third SSCP pattern (C) was only detected in one of the 48 samples, and subsequent sequencing showed that as well as the -1230G>A variant, there was a C to T change -1231bp of the transcription start site (Fig. 3.5 C). These results were confirmed by sequencing the reverse strand.

Fig. 3.5: Primer set 3 sequencing. The region of interest shows a G>A change explaining the change in SSCP pattern B. One individual (C) also showed a C>T change which was not replicated in any other samples.



3.3.3 Allele frequencies of variants found

A PCR/restriction digest assay was developed for the C>A change 1700bp 5' of the transcription start site. A forward oligonucleotide with a base pair deliberately changed (T to a G 1bp upstream of the variant) was designed in order to introduce a Alu I restriction enzyme recognition site (AGCT) when amplified. The C to A change would remove this site, thereby preventing cleavage of the DNA. Table 3.2 lists the optimised PCR and digest conditions for this assay, which was carried out as described in section 2.2.3. A similar 'forced' PCR was also designed for the G-1230A change, with an altered reverse oligonucleotide introducing a *Mae III* GTNAC restriction digest site that was abolished if the Adenosine was present. The C-1231T change abolishes a *Bbs I* restriction site, therefore bypassing the need to design a forced PCR.

In order to determine allele frequency, 192 individuals from the NPHS II study were genotyped for all three potential variants. Genotyping revealed that the C-1700A change observed in sequencing was not present in any of the individuals genotyped. Similarily, genotyping of the C-1231T possible variant showed no variation in the individuals tested. However, the G-1230A change was polymorphic with a rare allele frequency of greater than 1% and did not depart from Hardy Weinberg equilibrium (Fig. 3.6 and Table 3.3).

Fig 3.6: Reverse UV image of a MADGE gel showing the G-1230A polymorphism. A 2% gel is unable to resolve the 24bp fragment

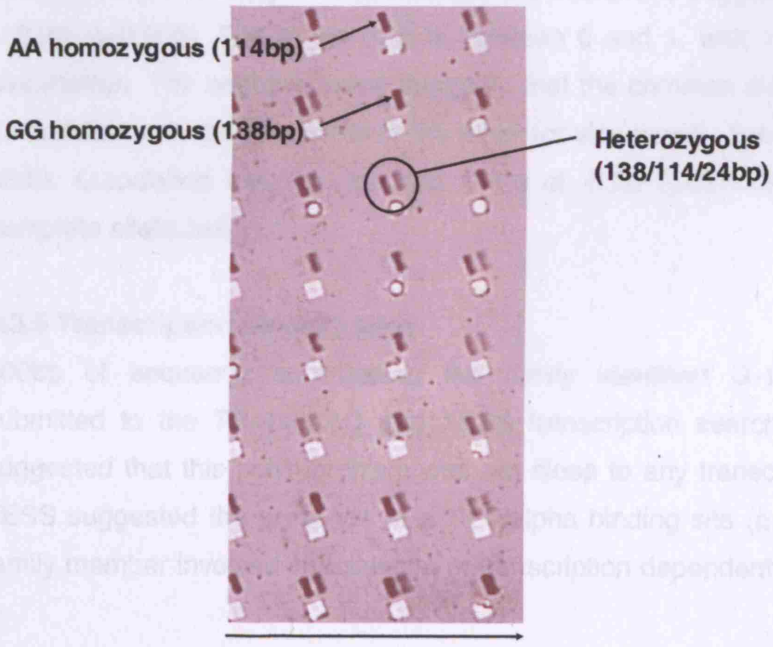


Table 3.3: Genotype data from the three potential variants found. Only G-1230A was polymorphic. The A379V genotype was included for comparison. 11= common homozygote, 12= heterozygote, 22= rare homozygote.

Potential SNP	Genotypes			Rare allele frequency	95% CI
	11	12	22		
C-1700A	189	0	0	NA	NA
C-1231T	185	0	0	NA	NA
G-1230A	109	65	8	0.22	0.18-0.27
A379V	**121**	**62**	**3**	**0.18**	**0.14-0.22**

3.3.4 Allelic association of G-1230A and A379V SNPs

Allelic association is a measure of the extent to which genomic sequences representing specific allele combinations (haplotypes) from a common ancestor have persisted over the course of evolution (Lander and Schork, 1994). Linkage disequilibrium (LD) is the non-random association between alleles at different loci and is used to define the relationship between SNPs: knowledge of the genotype at one SNP may predict the genotype of another SNP if the association is high enough (Crawford and Nickerson, 2005). In order to determine the level of LD between the functional A379V variant and the novel G-1230A SNP, two comparable statistical approaches were utilised, D' and Δ, on 400 individuals from the NPHS II study. The Δ between the two SNPs suggested a weak negative LD (Δ =-0.23, $p<0.005$). The range of Δ is between 0 and 1, with 1 representing strong allelic association. The negative value suggests that the common allele of one SNP is in allelic association with the rare allele of the other (or vice versa). However, D' indicated a strong allelic association between the two SNPs of -0.90 ($p<0.0005$), with +1/-1 representing complete allelic association.

3.3.5 Transcription binding sites

400bp of sequence surrounding the newly identified G-1230A polymorphism was submitted to the TRANSFAC and TESS transcription search systems. Both programs suggested that this polymorphism was not close to any transcription factor binding sites. TESS suggested the presence of a RORalpha binding site (a nuclear hormone receptor family member involved in activation of transcription dependent on intracellular cholesterol

(Boukhtouche et al., 2004)) 20bp 5' of the polymorphism but with low sequence homology. TRANSFAC noted an OCT-1 site (can repress and activate transcription under different conditions) with high homology, 50bp 3' of the G-1230A polymorphism (Fig 3.7).

3.4 Discussion

The most reliable system for the detection of polymorphisms within a population is still that of re-sequencing. However, the relative cost of sequencing means that other more cost-effective yet accurate methods are often used to 'pre-screen' DNA sections of interest (Suh and Vijg, 2005). Apart from SSCP, several other systems have been successfully used to identify variation in the genome, and are split between those utilising the property of conformational change and those reliant on the melting temperature changes of DNA (Suh and Vijg, 2005).

Cleavage fragment length polymorphism (CFLP) is a very similar method to that of the fellow conformation based technology of SSCP. CFLP relies on the fact that single strand DNA will form reproducible hairpin duplexes during self annealing. However, CFLP uses the enzyme *Cleavase I,* a structure specific enzyme that hydrolyses certain hairloop regions, giving a unique pattern of digestion when run on an agarose gel. CFLP is regarded as a more rapid and accurate method compared to SSCP, as well as enabling longer fragments of DNA to be analysed (Rossetti et al., 1997). However, getting a reproducible restriction pattern is often time consuming and difficult, requiring an optimum temperature and assay time-course for each strand under consideration (Suh and Vijg, 2005). A second method of conformational analysis is that of conformation-sensitive gel electrophoresis (CSGE) which is based on differences in conformation between homoduplex and heteroduplex double stranded DNA which exhibit distinct electrophoretic mobility (Ganguly et al., 1993). However, this method of detection is relatively low resolution and usually needs to be combined with SSCP in order to reach a detection rate of near 100% (Suh and Vijg, 2005).

Other techniques based on DNA melting have also proved to be popular pre-screening methods. Denaturing high-performance liquid chromatography (DHPLC) is a complicated system that is relatively low-throughput, but is useful in clinical diagnostics laboratories with limited numbers of samples (Xiao and Oefner, 2001; Suh and Vijg, 2005). More common are the related methods of denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman, 1979), and temperature gradient gel electrophoresis (TGGE) (Riesner et al., 1989) which rely on denaturing DNA unravelling in distinctive domains (not all at once) resulting in branched structures (Suh and Vijg, 2005). For DGGE,

polyacrylamide gels are poured with a gradient of denaturant from a high percentage at the anode to a low percentage at the cathode. With TGGE the gradient is that of temperature. When a DNA fragment reaches a denaturant concentration or temperature equivalent to the melting temperature of its lowest melting domain, branching occurs, lowering electrophoretic mobility. At higher melting domains, accuracy is greatly reduced since both strands may totally dissociate from each other, although this can be overcome with the introduction of a high melting temperature GC 'clamp' at the 5' end of one of the oligonucleotides (Sheffield et al., 1989). By applying a computer based program to predict optimal melting temperature (Lerman and Silverstein, 1987), and the use of HD, this method approaches almost 100% sensitivity from fragments up to 500bp in length (Suh and Vijg, 2005). There are serious disadvantages to this method relating to the high cost of GC-clamped oligonucleotides, the poor resolution of GC rich regions, and the use of toxic formamide.

In contrast to the methods described, SSCP has a number of advantages. The process itself is very simple and does not require the making of polyacrylamide gels if a capillary based system such as MegaBACE™ is used. The sensitivity of SSCP is over 80% for fragments shorter than 300bp (Hayashi and Yandell, 1993), and the cost of SSCP is comparatively low. In addition, standard sequencing equipment can be used and large number of samples analysed quickly (Ren, 2000). However, several distinct disadvantages do exist with SSCP and some of these problems are shared with the other described systems. The neighbouring base sequence around the mutation can have an effect on the degree of mobility shift observed (Glavac and Dean, 1993; Saitoh et al., 1998), and a number of electrophoretic conditions such as pH, gel matrix, temperature, and the addition of glycerol to the gel can influence the separation (Hennessy et al., 1998). Therefore reproducibility can sometimes be a problem, with some mutations potentially remaining undetected. Fundamentally all of these systems are unable to identify the position or nature of the change, therefore sequencing is required. For large screening efforts such as the detection of polymorphisms in the promoter of the PLA2G7 gene, SSCP provides one of the cheapest and most convenient methods currently available (Kristensen et al., 2001; Kozlowski and Krzyzosiak, 2001).

SSCP and HD analysis of the *PLA2G7* promoter (2Kb) revealed only three potential variants, of which a G to A change at position -1230 (rs12198175) was the only

polymorphic site when tested in 192 healthy individuals. Recent work by the GeneCanvas consortium (www.genecanvas.org) has established a central resource for polymorphisms found in a number of genes. The *PLA2G7* gene has been screened for novel polymorphisms using SSCP and re-sequencing, and the data published freely on the website. Apart from the already established variants of R92H, I198T and A379V which are described further in section 1.4.2.6, analysis confirmed two novel promoter polymorphisms, T-403C (rs1421378) and C-209G (rs9395208) which are in close positive allelic association (D' = 0.99), as well as two changes within exon 1 (G+108ex1nt/T) and 3' (T+107in1/C) of exon 1 (Fig 3.7). Subsequent analysis in the Athero*Gene* study has shown that the G+108ex1nt/T change was not polymorphic, and that the T+107in1/C change was in complete association with the T-403C variant (Ninio et al., 2004). The T-403C and the C-209G variants should have been detected within primer set 7. Retrospective analysis of the SSCP and HD patterns showed no variation, raising questions about the sensitivity of the method employed here. It's possible that the fragment length generated from this pair of oligonucleotides (454bp) was too long, reducing the sensitivity of the assay dramatically. Indeed, lengths of 200-300bp are regarded as optimal for the detection of variants by SSCP (Hayashi and Yandell, 1993). Other researchers have found that strands of DNA in the region of 500bp can be analysed with a reasonable degree of accuracy if combined with HD analysis (Kozlowski and Krzyzosiak, 2001), although it would appear that in this experiment SSCP and HD analysis was still unable to pick up two frequent polymorphisms.

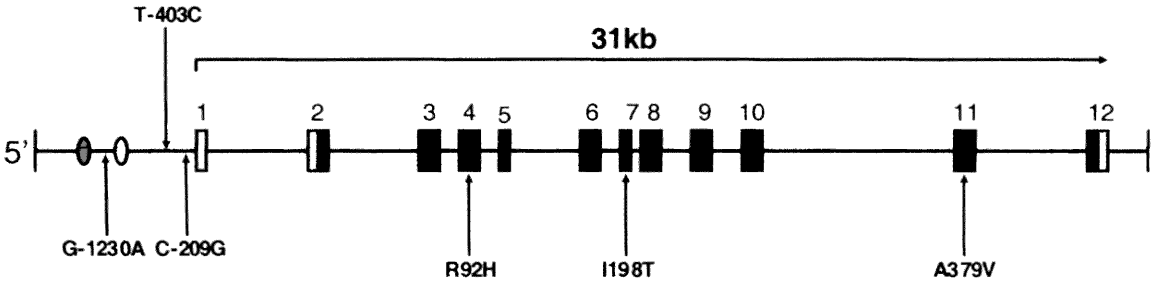
The G-1230A polymorphism appears to be in strong allelic association with the activity altering A379V variant when D' is considered. The Δ of these two SNPs suggested a much weaker, negative association. One of the potential reasons for this discrepancy could be that the two polymorphisms are relatively rare with regards to allele frequency (0.18 and 0.22 for A379V and G-1230A, respectively). Delta calculations are themselves sensitive to fluctuations in allele frequency (in particular if both SNPs are rare)(Devlin and Risch, 1995). D' meanwhile is more stable where allele frequencies are comparatively rare (Zondervan and Cardon, 2004), and is regarded as a robust measure of LD across a range of conditions (Devlin and Risch, 1995; Hedrick, 1987).

The SSCP work and association studies carried out in this thesis were conducted before the GeneCanvas website published detailed information regarding the other SNPs found in

the promoter of *PLA2G7*. The proliferation of more comprehensive SNP information taking into account allele frequencies and LD in a range of different populations has enhanced the screening of the *PLA2G7* promoter, namely by displaying two other novel SNPs that would not have been originally found by the author in the present study. Work conducted by Ninio *et al.* has subsequently found that the T-403C and C-209G polymorphisms are in very tight negative LD (as measured by D') with the A379V variant, and that neither polymorphism significantly affected Lp-PLA2 enzyme activity or CHD risk (Ninio et al., 2004). Their data firmly suggested that the associations with risk and activity seen in haplotype analysis, was the result of the A379V variant only. Therefore, the two promoter polymorphisms were not genotyped and considered for further analysis with regards to association studies in this thesis. Initial analysis of the G-1230A polymorphism has suggested that this polymorphism does not interfere with any known transcription binding sites as determined by TESS and TRANSFAC. This does not however, mean that the G-1230A polymorphism is inconsequential with regards to the rate of *PLA2G7* transcription, since the systems used to determine transcription binding sites are still in development, and do not provide concrete evidence either way of the existence of transcription binding domains.

That all of these SNPs are in tight LD with the A379V variant (Fig 3.7), makes it difficult to determine if any of the promoter SNPs exhibit an independent function of their own. Aim 3 of this thesis was designed to determine the potential functional role (namely the effect on the rate of transcription) of the novel G-1230A SNP independently of the A379V variant. Chapter 4 of this thesis would hope to address whether the identified G-1230A SNP was itself associated with markers and risk of atherosclerosis.

Fig 3.7: Schematic of the novel G-1230A promoter SNP in relation to other previously identified SNPs. The table below represents LD measured by D'. The data is compiled from the GeneCanvas database and results obtained in this chapter (in bold).



Putative ROR-alpha site

Putative OCT-1 site

	C-209G	R92H	I198T	A379V
T-403C	+0.99	+0.94	+1.00	+1.00
C-209G		+0.79	-0.80	-0.85
R92H			-0.84	-1.00
I198T				-1.00
G-1230A				**-0.90**

CHAPTER 4

ASSOCIATION OF *PLA2G7* POLYMORPHISMS WITH Lp-PLA2 ACTIVITY, RISK AND MARKERS OF ATHEROSCLEROSIS

4.1 Gene variants and their role in investigating the pathogenesis of CHD

4.1.1 Methods employed to study complex disease

The main aims of genetic research into CHD relate to identifying disease causing genetic variants, hopefully leading to a further understanding of the pathological processes involved in atherosclerosis (Talmud and Humphries, 2002). However, the genetics of multifactorial disorders such as CHD are complex, as susceptibility does not always follow simple Mendelian monogenic inheritance and doesn't exhibit classic Mendelian recessive or dominant inheritance attributable to a single gene locus (Lander and Schork, 1994). In order to study the complicated gene-gene and gene-environment interactions that characterise the genetics of CHD, for common methods have been proposed to study complex disorders: association studies, Transmission Disequilibrium Tests (TDT), linkage analysis, and allele-sharing studies.

4.1.1.1 Association studies

Association studies performed on samples of the population investigate whether genetic variation, within or close to a gene of interest, is associated with inter-individual differences in the intermediate phenotype (biochemical or clinical), or with increased risk of disease. This may occur because the genetic variation is causal (for example a sequence change leading to an amino acid substitution) or because of linkage disequilibrium (LD) with another sequence change which is causal, that lies elsewhere in the gene (Suh and Vijg, 2005). Two different samples are typically studied in association studies. Large prospective cohorts are powerful at measuring risk associated with a specific gene variant, as they measure risk over time in a population. These studies are also useful in studying gene-environment interactions because of their longitudinal design measuring exposure before disease onset. The population is often carefully selected at baseline to have similar characteristics regarding non-modifiable traits such as age or ethnic origin and does not pre-select individuals with CHD (Rothman and Greenland, 1998). Unfortunately, prospective studies take a relatively large amount of time to implement since populations are recruited at baseline; therefore the case-control study was developed as an alternative and less costly design. Case-control studies allow for the comparison of risk factors (environmental or genetic) between groups of unrelated subjects with and without disease, or in the case of placebo-trials, those individuals taking a drug versus a placebo group (Zondervan and Cardon, 2004). Case-control studies are easier to conduct since they do not require long-term follow-up and results can be made available quickly. They also allow

a more precise characterisation of outcome, as well as greater statistical power to detect associations than would be feasible with a prospective design, by ensuring all cases are scanned properly for a particular phenotype. In this respect case-control studies are useful to study the association between gene variants and various intermediate phenotypic measures. The main disadvantage of these studies is that errors in the measurements of exposures, (e.g. age, ethnic origin) can differ systematically between cases and controls, and therefore give misleading results (Farrall and Morris, 2005; Zondervan and Cardon, 2004). The fact that cross-sectional studies are carried out at a single time point means that knowledge of prior events is often limited. Because of this, one can only state that there is a statistical association between the factor of interest (gene polymorphism) and disease, and not that the factor is likely to cause disease.

Both prospective and case-control studies are effective tools for studying complex traits because they have the statistical power to detect associations in genes with small effects (Risch and Merikangas, 1996; Long and Langley, 1999). However, several difficulties still exist with these types of studies, in particular a lack of reproducibility of significant associations (Hirschhorn and Altshuler, 2002; Ioannidis et al., 2001). This type of problem usually arises with an over-estimation of the effect of a genetic polymorphism in several modestly sized studies. Eventually a large study is assessed and a meta-analysis conducted, resulting in a greatly reduced genetic effect size (Farrall and Morris, 2005). This observation could be affected by the phenomenon of publication bias, where only those positive associations are published (Colhoun et al., 2003). Secondly, many of the studies previously tested have failed to have adequate numbers of cases to be sufficiently powered to examine disease genetics. Relative risk arising from genetic variation may be small, therefore performing association studies with modest sample sizes could lead to an over-estimation of the size of genetic effects (Colhoun et al., 2003; Farrall and Morris, 2005). Small association studies are also prone to random chance (also termed type I error) often producing significant conclusions with wide confidence intervals (Cardon and Bell, 2001).

A second important consideration is that there may be variation in the underlying association between genotype and the outcome between different populations studied. This could be caused by different disease-causing alleles predominating in different populations or variation in LD existing between marker and disease (Colhoun et al., 2003). LD is dependent upon population history and on the genetic makeup of the

founders of that population; therefore inconsistencies can occur if populations are mixed (Hirschhorn et al., 2002). To avoid these problems it is important to sample suitably homogeneous populations that try to represent a similar population group (i.e. based on ethnicity or geographical position). An additional reason for the discrepancies found in some studies is that gene-gene and gene-environment interactions may differ between populations. Variants may only manifest their effects in populations with a particular genetic or environmental background, indicating that replication studies should take carefully into account these impacts (Redden and Allison, 2003; Talmud and Humphries, 2002). In addition, many studies do not try to stratify by risk factors or look for evidence of a gene-environment interaction therefore weakening the ability to detect significant associations.

Many of the problems listed here can be avoided by using accurately phenotyped, homogeneous large association studies that are powered to detect the small to moderate effects of common functional SNP variants. It has also been proposed that statistically significant associations should be reproduced in several independent samples (Humphries and Donati, 2002), and that meta-analysis of all published data could provide a quantitative estimate of the genetic risk factor and disease (although these analyses are often subject to publication bias)(Ioannidis et al., 2001).

4.1.1.2 Transmission disequilibrium test (TDT)

The TDT method of analysis can be conceived as a form of association study which avoids the possibility of spurious positive results produced by population stratifications present in conventional association studies (Lander and Schork, 1994). In the TDT, the transmission of alleles from heterozygous parents is analysed and comparison made between those alleles which are transmitted to affected offspring (these represent the 'cases') and those which are not (the untransmitted alleles provide a population matched control). If an allele is transmitted to an affected subject more often than not then this is robust evidence for an association with the disease.

4.1.1.3 Linkage analysis

Over the last few decades, the most popular study design for associating genes and gene variants with human disease phenotypes has been linkage analysis. Genetic linkage describes the association of two or more loci on the same chromosome with limited recombination between them. This is not the same as LD which statistically describes the non-random association of alleles at two or more loci. With linkage analysis, the whole genome is scanned at numerous, evenly spaced polymorphic loci

in a number of families with a history of the disease (pedigrees or sib-pairs), to identify chromosomal regions linked to disease and related factors (Farrall and Morris, 2005). It involves constructing a disease model to explain the inheritance pattern of the disease, by comparing the observed segregation of gene markers and trait in affected pedigrees. Linkage is the method of choice for studying simple Mendelian traits in monogenic disorders because the allowable models are few and easily tested (Suh and Vijg, 2005). However, application to complex, multifactorial disorders are problematic, as the genetic analysis may be confounded by incomplete penetrance, phenocopy, genetic heterogeneity, polygeneic inheritance, absence of large multi-generated pedigrees and mis-inheritence (Lander and Schork, 1994). Linkage is well powered to detect a major gene effect with few confounders, but not to detect genes of modest effect. Nonetheless, a successful application of linkage analysis using 513 western families has located the risk of MI to a single region on chromosome 14 (Broeckel et al., 2002), although the exact gene (s) has yet to be fully determined.

4.1.1.4 Allele-sharing

A final method for studying complex diseases is the allele-sharing approach. This involves studying affected relatives (or siblings) in a pedigree to see how often they inherit identical copies of the region from a common ancestor (Kurtz and Spence, 1993) and to obtain statistical evidence that the inheritance pattern of the chromosomal region is not consistent with random Mendelian segregation. This method typically applies to a single generation of disease sufferers. It is independent of the pattern of disease inheritance and therefore is frequently used in the analysis of a complex disorder with a late onset, such as CHD risk.

4.1.2 Common gene variants and association analysis

In the past, the aim of linkage studies was to define regions of the chromosome where sequence markers (SNPs) were correlated to chronic diseases with a genetic component. The search for causative markers could then be limited to the chromosomal region associated with disease (Kruglyak, 1999). While in the past linkage studies were carried out in a limited number of sequence variants or genes, the advent of the Haplotype Map (HapMap) has provided a global coverage of the genome with SNP markers. The HapMap project has been able to genotype in the region of 1.1 million SNPs in 270 individuals of clearly defined ethnic origin, with the goal of

developing a map of non-redundant tagging SNPs (Thorisson et al., 2005). These SNPs may then be able to capture most of the common genetic variants contributing to complex disease. This approach is unbiased and allows a comprehensive genome-wide survey, but is limited by a lack of knowledge concerning LD heterogeneity across different populations (population stratification), and the necessity of large sample sizes to avoid multiple testing errors (Suh and Vijg, 2005). These problems can be further exacerbated if the disease gene variant occurs at a low frequency. The inherent cost associated with genotyping SNPs across a large section of genome is also high. An alternative approach is that of the indirect/direct candidate gene pathway which is based on knowledge of the disease phenotype, genetic studies in model organisms, or location (as defined by a linkage study) (Tabor et al., 2002). Indirect candidate association studies are those that utilise pre-defined tagging SNPs across a gene (or region) of interest to investigate the genetic aetiology of complex diseases such as CHD. The concept of indirect candidate association is discussed in more detail in chapter 7.

Direct candidate associations

The progressive improvements in SNP discovery methods such as sequencing mean that in the distant future all possible genetic variation within gene coding and regulatory regions could be genotyped in every individual of an association study, in order to determine the functional SNP. Until that time, direct association studies are limited to candidate SNP analysis, where the association between putative functional variants and disease risk is tested (Suh and Vijg, 2005).

As discussed in chapter 3, functional SNPs can be characterised as those that are within coding regions and lead to changes in amino acid sequence, or regulatory SNPs that modulate expression or splicing of the gene. It is often difficult to assess SNP function on the basis of nucleotide sequence alone, and particularly when SNPs do not alter an amino acid or disrupt a well known motif that affects protein function or structure. An important challenge of any study is to define the variants that are functionally implicated in the disease, since only a small subset of variants will confer moderate to small effects on the phenotypes that are related to disease risk. Since many of these variants are low in frequency, studies have to be sufficiently powered in order to detect any potential associations. Making this approach even more difficult is that those individuals most susceptible to complex diseases such as CHD, are often at elevated risk due to the combined effects of several susceptibility alleles (a haplotype effect) (Pritchard and Cox, 2002). This makes it important to determine as precisely as

possible the functional SNPs before proceeding with a candidate SNP approach. Added to this, is the complication of gene-gene and gene-environment interactions that may affect the statistical significance of any association obtained.

For this thesis, five studies of differing design were employed to investigate the association of the putatively functional A379V and newly discovered G-1230A genotype with CHD risk, Lp-PLA2 activity, and markers of atherosclerosis. All five studies are susceptible to the same criticisms listed here, however, it was hoped that the size of the studies, and accuracy of the phenotyping would help confirm the role of the Lp-PLA2 enzyme with regards to atherosclerosis. The Northwick Park Heart Study II is a powerful prospective study encompassing over 3000 individuals. This study was used to investigate further the association of the A379V variant with risk, as well as Lp-PLA2 activity. Similarly, the nested case-control EPIC-Norfolk study of over 1000 cases matched to 2000 controls would provide a powerful study for investigating the association of A379V genotype with Lp-PLA2 activity and CHD risk. The G-1230A polymorphism was genotyped in the HIFMECH study encompassing over 500 cases (men who have experienced an MI before the age of 60) and 500 controls in order to investigate the association of this polymorphism with CHD risk. The University College London Diabetes and Cardiovascular Study (UDACS), is a cross-sectional study (n>1000) and was used to investigate a cohort of high risk patients with diabetes mellitus and to characterise the relationship of Lp-PLA2 activity and genotype (A379V and G-1230A) with LDL oxidation and atherosclerosis. The final study investigated was the Bassingbourne II study (Big Heart II, BH2): a follow-up design that accurately measured adiposity and lean muscle mass before and after 12 weeks of physical training. The aim of this study was to investigate novel roles of Lp-PLA2 with regards to changes in body composition.

Firstly, by genotyping the *PLA2G7* A379V variant in two large, well powered studies, it was hoped that the association of *PLA2G7* genotype with CHD risk could be replicated, hence confirming the causality of the Lp-PLA2 enzyme in atherosclerosis (Abuzeid et al., 2003; Ninio et al., 2004). The addition of Lp-PLA2 activity measures would further enhance the understanding of the functionality of this variant and enzyme with regards to CHD. The HIFMECH study would be used to determine if the novel G-1230A variant was associated with CHD risk. And finally the UDACS and BH2 study would enable the investigation of the Lp-PLA2 enzyme under different pathological stimuli in the form of Diabetes and exercise training. It was hoped that these range of studies would push forward the understanding of this enzyme's relationship with atherosclerosis.

4.2 *PLA2G7* genotype associations with CHD and enzyme activity in a large prospective study.

4.2.1 Introduction

The potential pro-/anti- atherogenic properties of the Lp-PLA2 enzyme have been discussed in chapter 1, and are supported by contrasting evidence in a variety of *in vitro* and *in vivo* systems (sections 1.4.2.8 and 1.4.2.9). So far, epidemiological data has not been able to full clarify the pro- and anti-atherogenic role of Lp-PLA2. Both activity (Blankenberg et al., 2003; Oei et al., 2005; Winkler et al., 2005) and mass (Ballantyne et al., 2004; Blake et al., 2001; Packard et al., 2000) of the enzyme in Caucasian populations have been shown to be consistent risk markers for CHD, independent of traditional risk factors, although it is still unclear whether the association of Lp-PLA2 enzyme with CHD risk represents a direct causal relationship of enzyme with CHD.

Within the gene encoding the Lp-PLA2 enzyme (*PLA2G7*), a loss-of-function mutation present only in the Japanese population exists (V279F), and has been reported to be associated with an increased risk of CHD (Yamada et al., 1998), supporting an *anti-atherogenic* action of this enzyme. In order to relate the variability within the *PLA2G7* gene to atherosclerosis within Caucasian subjects, several other variants have also been detected, with the Alanine to Valine change at position 379 (exon 11) being found to be potentially functional (Kruse et al., 2000). Subsequent *in vitro* studies showed a 2-3 fold decrease in the affinity of Valine 379 Lp-PLA2 recombinant protein for its substrate PAF (Kruse et al., 2000). The altered amino acid resides close to the important catalytic triad governing enzyme activity, further suggesting a functional role (Karasawa et al., 2003). Previous data from our laboratory has shown that V379 homozygotes were protected from CHD in the HIFMECH case-control study (Abuzeid et al., 2003). The previous association of this genotype with lower Lp-PLA2 PAF substrate affinity would therefore support a *pro-atherogenic*, causative role for Lp-PLA2 in CHD.

Ninio *et al.* have investigated the *PLA2G7* A379V gene variant further, as well as several other known non-synonymous and promoter variants, in the Athero*Gene* study of 1318 CHD patients and 485 controls (Ninio et al., 2004) (the common SNPs of the *PLA2G7* gene are shown in Fig. 3.7). With respect to case-control status and clinical outcome, the 379V allele was found to be independently protective against the

development of CHD, supporting the HIFMECH study (Abuzeid et al., 2003). However, in contrast to data produced by Kruse *et al.* (Kruse et al., 2000), those homozygous for the 379V allele showed significantly *higher* plasma Lp-PLA2 activity towards hydrolysing PAF substrate compared to AA and AV individuals, suggesting an *anti-atherogenic* role for Lp-PLA2 in atherosclerosis.

In light of the previously published epidemiological data, one of the aims of this thesis was to investigate whether the A379V polymorphism in the *PLA2G7* gene was associated with CHD risk and enzyme activity in a large prospective population of over 3000 healthy middle aged men (of which there are over 200 verified CHD cases). If the A379V polymorphism was found to be consistently associated with CHD risk across a number of studies, then a causal relationship of the enzyme with atherosclerosis may exist. However, to ascertain whether this relationship was pro or anti atherogenic, it was also important to relate the putatively functional variant to Lp-PLA2 enzyme activity. This section of chapter 4 describes the associations of the *PLA2G7* A379V variant, Lp-PLA2 activity, and CHD risk in the NPHS II prospective study. In a small subset of this population, specific activity measures (activity/mass) were taken and these were also investigated with regards to A379V genotype.

4.2.2 Materials and Methods

4.2.2.1 The Second Northwick Park Heart Study II

From April 1989 to April 1994 (median 10.2 years of follow-up), 3012 healthy European, Caucasian men, aged 51-60 years, registered with 9 general medical practices in the UK, were recruited for prospective surveillance (Miller et al., 1995; Miller et al., 1996). Follow-up is still continuing with 26 individuals having been lost to follow-up. All eligible subjects were free of a history of unstable angina, MI or evidence of silent infarction, coronary surgery, aspirin or anticoagulant therapy, cardiovascular disease, malignancy (except skin cancer other than melanoma), or any condition precluding informed consent. Participants were asked to attend a clinic in a non-fasting state, having been instructed to avoid heavy meals before examination and to refrain from smoking and vigorous exercise from midnight of the previous night. DNA was obtained for 2700 people whose baseline characteristics were not significantly different from the whole study group (Humphries et al., 2001).

Participants answered a questionnaire for previous medical history and smoking habits, and were classified as never smokers, ex-smokers (cessation for minimum 1 year) and current smokers. A standard electrocardiogram (ECG) was recorded and coded according to Minnesota criteria. This resulted in the exclusion of 42 men with changes indicative of MI. Participants were recalled annually for interview and repeat plasma analysis. A routine ECG was repeated at the sixth examination. Endpoints for CHD were fatal and non-fatal MI based on WHO criteria (World Health Organisation, 1975), silent MI or coronary revascularization procedures and sudden unexplained death. These events occurred from 64 days to 13 years of follow up with clinical information for each event being collected and submitted to an independent assessor who assigned qualifying events to the appropriate category.

Weight, height and systolic blood pressure (SBP) measurements were recorded and venous blood samples were collected for plasma and DNA analysis. Blood pressure was recorded twice with a random zero mercury sphygmomanometer after the subjects had been seated, and results were averaged for statistical analysis. Height was recorded to the nearest 0.1cm and weight to the nearest 0.1kg. Body mass index (BMI) was calculated as weight/height^2. A 5 ml sample of venous blood was taken using a Vacutainer (Becton Dickinson, Cowley, Oxford, UK) from each patient and transferred to plastic screw-cap vials, which were stored at -40^{0}C pending analysis. Cholesterol

and triglyceride concentrations were measured using automated enzyme procedures with reagents from Incstar (Humphries et al., 2001).

4.2.2.2 A379V genotyping

Oligonucleotides for the A379V polymorphism were designed using Primer 3 software, and are shown in Table 2.1, along with the relevant restriction enzyme conditions required. Section 2.2 of the material and methods chapter describes the complete PCR and digestion process.

4.2.2.3 Lp-PLA2 activity and mass measurements

Subset analysis of mass and activity by GSK R&D

Initially, a small subset of the NPHS II sample was assayed for activity and mass at the request of researchers at the GlaxoSmithKline Research and Development facility Stevenage using two methods that have been extensively trialled in epidemiological studies. The measurement of Lp-PLA2 activity and mass enables specific activity to be investigated, which takes into account differences in enzyme mass. All measurements were carried out by researchers at GlaxoSmithKline R&D.

i) Lp-PLA2 mass

Lp-PLA2 mass was measured using the DiaDexus PLAC® kit (DiaDexus Inc., USA). The PLAC test is a sandwich enzyme immunoassay previously described by Caslake *et al.* (Caslake et al., 2000) for the direct measurement of Lp-PLA2 concentration in human plasma and serum (Dada et al., 2002). The monoclonal anti-Lp-PLA2 antibody (2C10) was immobilised in microtitre plate wells. In the presence of high detergent concentration, the test plasma samples (diluted 1:10), in triplicate, were allowed to react with the immobilised antibody together with a second, biotinylated monoclonal Lp-PLA2 antibody (4B4), on a plate shaker, at room temperature, for 1 hour. As a result, Lp-PLA2 was captured between the immobilised and the solution-phase biotinylated antibodies. The wells were then washed to remove any unbound antibody. Eu-labelled streptavidin was added, which binds to the biotin of the secondary antibody, and left to incubate on a plate shaker, at room temperature, in the dark, for 1 hour. After washing, enhancement solution was added which promotes dissociation of Eu3+ from the solid phase bound Eu-labelled streptavidin to form a homogenous and highly fluorescent Eu-(2-NTA)3(TOPT)2-3 micellar chelate solution. Fluorescence was measured using the Delfia Research Fluorometer, which is directly proportional to the concentration of Lp-PLA2 present. A standard curve of recombinant Lp-PLA2 was included on each plate from which plasma Lp-PLA2 was determined. Linearity was typically $R^2 > 0.99$. The

assay was carried out according to manufacturers' instructions with a manufacturers reported minimum detection limit of 1.3ng/ml, and maximum intra-assay CV of 5.8% and inter-assay CV of 8.7%.

ii) Lp-PLA2 activity

Lp-PLA2 activity was measured in duplicate from citrated plasma stored at -80°C by the trichloroacetic acid precipitation procedure in 96-well plates described previously (Blankenberg et al., 2003; Tselepis et al., 1995). The GSK in-house assay used the same principle but with some laboratory specific alterations that were not disclosed to the author. 5μl of the plasma sample (plasma is diluted 1:100 in 90 μl of Lp-PLA2 assay buffer (pH 7.4) containing 4.2mmol/L HEPES, 137 mmol/L NaCl, 2.6 mmol/L KCl, and 2mmol/L EDTA) was aliquoted into each well of a 96-well Costar plate. 100μl of 100μM PAF solution (made up of 3H-PAF at 0.4μM and cold [C16] PAF at 99.6μM) was then added to each well and incubated at 21°C for 5 min. 50μl of ice cold BSA solution (50mg/ml) was added to each well, thoroughly mixed, and incubated in a refrigerator for 5min. 25μl of 56% Trichloroacetic acid (TCA, Sigma Aldrich) solution was added to the plate and incubated in the fridge for a further 15min. After these incubations the plates were centrifuged at 6000g for 15min at 4°C and a 45μl aliquot transferred to a fresh polystyrene plate. 200μl of MicroScint-20 scintillation cocktail (Perkin Elmer Biosciences, Boston, MA) was added to each well, the wells sealed, and vortexed for 10min. A TopCount scintillation counter (Perkin-Elmer, USA) was used to assay the radioactivity for 2min. Researchers calculated the Lp-PLA2 activity (nmol/min/ml) from the following formula:

$$\text{Lp-PLA2 activity} = 160 * (CPM_{45\mu l\text{-supe}} - CPM_{Blanks})/(CPM_{10\mu l\text{-spiking}} - CPM_{Blanks})$$

Where $CPM_{45\mu l\text{-supe}}$ is the average count from each sample

CPM_{Blanks} is the average count of the Blanks

$CPM_{10\mu l\text{-spiking}}$ is the average count of the Total counts

A mean intra-assay CV of 4.0% was observed for the NPHS II samples tested, and a mean inter-assay CV of 25%.

Activity measures in the entire sample

Activity measures for the entire NPHS II sample were kindly assayed by Ewa Ninio's group based in France (INSERM-Paris), using the 96-well plate format trichloroacetic acid precipitation procedure (Blankenberg et al., 2003; Tselepis et al., 1995). The

procedure was slightly different to the method used by GSK and contained the following steps: Plasma samples were diluted 1:100 in Lp-PLA2 assay buffer (pH 7.4) containing 4.2mmol/L HEPES, 137 mmol/L NaCl, 2.6 mmol/L KCl, and 2mmol/L EDTA to a final volume of 90 μl. After pre-incubation at 37 °C, 10 μl of 50 μM 3H-PAF (NEN-Dupont de Nemour, Boston, MA; specific activity, 81,000 ± 2,000 dpm/nmol) was added. Samples, in duplicate, were then incubated for 10 min at 37°C. The reaction was stopped in an ice bath, and BSA (16.7mg/ml) added to bind to any un-reacted 3H-PAF and incubated in a refrigerator for 5 min. TCA (8%) was added and incubated in a refrigerator for 15 min and the plate centrifuged as in the previous protocol. After precipitation, the radioactivity was assessed in the supernatant using a Optiphase Hi-Safe 3 scintillation counter (Perkin-Elmer, USA). The activity of Lp-PLA2 was expressed in nmol PAF hydrolysed/min per ml of plasma. A pool of control plasma (n = 10) served as an internal standard for all measurements. For the entire NPHS II study the mean intra-assay CV was 3.7% and the mean inter-assay CV was 34.3%.

4.2.2.4 Statistical analysis

Data was entered onto an EXCEL spreadsheet (Microsoft) and tested for deviation from Hardy-Weinberg equilibrium by using a χ^2 test. Statistical analysis was performed by Jackie Cooper, our statistician, using STATA (Intercooled STATA Version 8.0, STATA Corp.). Continuous variables are expressed as means ± 1 standard deviation (±1SD) for normally distributed variables, or geometric means and approximate SD for variables where normal distribution was reached after a log or square root transformation. Normality was considered graphically with QQ-norm plots and formally via a Kolmogorov-Smirnov test. A univariate step-wise regression model was subsequently applied to assess contributors to variance for Lp-PLA2 activity.

Analysis of variance (ANOVA) was performed to compare differences in continuous variables by genotype, and transformed response variables were used as appropriate. Relative risk of CHD by genotype and Lp-PLA2 activity in the NPHS II study was considered using Cox-proportional hazards survival analysis (Hazard Ratio, HR). Hazard ratios are a form of relative risk that represent a summary of the difference between two survival curves of CHD cases compared to controls over a period of follow-up. Cox regression models assume that the hazards for the groups being compared are constant at each interval of time. These models use a computable algorithm to summarise the relative risks of death across all time intervals into a single figure. All models were adjusted for age and differences in the baseline hazards in the nine practices considered using the strata function. Additional adjustments are also

listed in the text. Hazard ratios are presented along with 95% confidence intervals and p-values <0.05 were considered as significant. Survival analysis is also shown graphically using Kaplan-Meier survival plots which show the cumulative survival percentage plotted against follow-up of the NPHS II study. Bonferroni correction for multiple comparisons was not applied since all analysis was based on *a priori* hypothesis. Any novel associations that were borderline significant were subsequently interpreted with caution.

4.2.3 Results

4.2.3.1 Baseline characteristics of the NPHS II cohort

Table 4.2.1 lists the baseline characteristics by CHD status for the NPHS II study in those individuals with complete Lp-PLA2 measurements. Those men with CHD had higher systolic BP (p=0.0004), cholesterol (p=0.0005), LDL (p=0.03), and TG levels (p<0.0001). Individuals with CHD also had lower levels of HDL (p=0.04), were more likely to be smokers (p=0.03), and had higher levels of inflammatory markers such as fibrinogen (p=0.01) and CRP (p=0.02). Lp-PLA2 activity measures while higher in those individuals with CHD (49.2± 16.4 nmol/min/ml) compared to those without (48.1± 15.5 nmol/min/ml) did not reach statistical significance (p=0.36).

4.2.3.2 Association of Lp-PLA2 activity with baseline characteristics

Table 4.2.2 lists the correlation of Lp-PLA2 activity with other risk factors measured in the NPHS II study. Analysis was based on averages of baseline and year 1 results for all variables (except CRP, LDL and HDL where measurements are available only in a single year). There were significant correlations of Lp-PLA2 activity with lipid measures such as; cholesterol (r=0.31, p<0.0001), TG (r=0.20, p<0.0001), LDL (r=0.28, p<0.0001), and HDL (r=-0.25, p<0.0001). There were also weak but significant correlations of Lp-PLA2 activity with BMI (r=0.07, p=0.001), systolic BP (r=0.08, p<0.0001), diastolic BP (r=0.07, p=0.001), and CRP (r=0.04, p=0.04).

In a stepwise regression model of baseline measures, Lp-PLA2 was found to be independently associated with cholesterol, HDL, fibrinogen, and smoking status. Together they explained 14.6% of the variability in Lp-PLA2 activity measures. Table 4.2.3 shows the baseline characteristics of the NPHS II study by quartiles of Lp-PLA2 activity. Higher Lp-PLA2 activity was significantly associated (after suitable adjustment for cholesterol, HDL, fibrinogen and smoking) with LDL (p=0.01), HDL (p<0.0001) and cholesterol levels (p<0.0001). Those with higher levels of Lp-PLA2 activity were also more likely to be smokers (p=0.02), although no significant difference in Lp-PLA2 activity was observed between current (48.52± 16.2 nmol/min/ml) and non (48.10± 15.3 nmol/min/ml) smokers (p=0.56). There was a non-significant trend for higher systolic BP with higher Lp-PLA2 activity (p=0.08 after adjustment).

4.2.3.3 Association of Lp-PLA2 activity with CHD risk

As discussed in the introduction of this chapter, prospective cohorts enable CHD risk to be studied over a period of time using Cox regression models (Hazard Ratio) and the Kaplan-Meier survival graph. Unfortunately, quartiles of Lp-PLA2 activity showed no statistically significant association with relative risk in the NPHS II study after adjustment for age and practice (model 1)(Table 4.2.3). However, there did appear to be a non-significant trend (p=0.37) towards higher CHD risk in those individuals with higher Lp-PLA2 activity (model 1). Further adjustment for age, practice, systolic BP, HDL, LDL, Cholesterol, fibrinogen and smoking (model 2) are also shown in table 4.2.3. When tertiles of Lp-PLA2 activity were also considered, a trend was still apparent in both models but still failed to reach statistical significance (p=0.34 for model 1, and p=0.48 for model 2)(Table 4.2.3)

By plotting the cumulative survival percentage of the NPHS II sample against follow-up (in time) it is possible to display the cumulative probability of an individual remaining free of CHD at any time after baseline. Survival 'curves' for individuals in each quartile of Lp-PLA2 activity are shown in figure 4.2.1. Although there appears to be little difference between quartiles with regards to survival up to 15 years, it does appear that after this time those with higher Lp-PLA2 activity have a lower probability of survival. The right hand side of a Kaplan-Meier plot often suffers from small numbers caused by drop-outs and deaths, possibly explaining the large drop off in probability of survival seen after 15 years. Interestingly, when considering the mean age of CHD onset, there was no significant difference in mean age by quartiles (p=0.96) of Lp-PLA2 activity (Table 4.2.4).

Table 4.2.1: Baseline characteristics (mean± 1SD) of the NPHS II study by CHD status in those individuals with Lp-PLA2 activity data

Trait	No CHD N=2129	CHD N=185	P value
Age, years	56.1 (3.5)	56.5 (3.5)	0.12
BMI kg/m^2 *	26.1 (3.3)	26.3 (3.3)	0.41
Systolic BP, mmHg *	134.1 (16.9)	138.7 (18.1)	0.0004
Smoking (%)	590 (27.7)	65 (35.1)	0.03
LDL, mmol/L	3.97 (0.96)	4.19 (0.84)	0.03
HDL, mmol/L *	0.81 (0.24)	0.77 (0.25)	0.04
Cholesterol, mmol/L	5.63 (0.93)	5.88 (0.94)	0.0005
Triglyceride, mmol/L *	1.75 (0.84)	2.02 (0.98)	<0.0001
Fibrinogen, g/L *	2.69 (0.46)	2.79 (0.44)	0.01
CRP, mg/L *	2.84 (3.31)	3.54 (4.17)	0.02
Lp-PLA2 activity, nmol/min/ml *	48.1 (15.5)	49.2 (16.4)	0.36
V allele freq (95%CI)	0.18 (0.17-0.20)	0.20 (0.16-0.24)	0.46

*Natural log geometric mean. SD is approximate

Table 4.2.2 Correlation of Lp-PLA2 activity with other risk factors

	N	Correlation	P value
Age	2314	r= -0.01	p=0.49
BMI[1]	2314	r= 0.07	p=0.001
SBP[1]	2314	r= 0.08	p<0.0001
DBP	2314	r= 0.07	p=0.001
Cholesterol	2304	r= 0.31	p<0.0001
Triglyceride[1]	2305	r= 0.20	p<0.0001
Fibrinogen[1]	2306	r= 0.02	p=0.38
CRP[1]	2185	r= 0.04	p=0.04
LDL	1554	r= 0.28	p<0.0001
HDL[1]	1651	r= -0.25	p<0.0001

[1]log-transformed
Lp-pla2 is square root transformed before analysis.

Table 4.2.3: Baseline characteristics (mean± 1SD unless otherwise stated) of the NPHS II study separated by Quartiles of Lp-PLA2 activity.

	Quartile1 <39	Quartile 2 39-47.9	Quartile 3 48-58.2	Quartile 4 >58.2	P value (unadjusted)	P value (adjusted)[+]
N=	579	578	579	578		
BMI kg/m^2 *	25.8 (3.3)	25.9 (3.1)	26.3 (3.4)	26.4 (3.3)	0.005	0.82
Systolic BP, mmHg *	133.5 (17.3)	132.9 (17.0)	134.6 (16.9)	136.7 (16.8)	0.0007	0.08
Smoking (%)	157 (27.1)	150 (26.0)	180 (31.1)	168 (29.1)	0.23	0.02
LDL, mmol/L	3.56 (0.93)	3.89 (0.87)	4.09 (0.84)	4.36 (0.99)	<0.0001	0.01
HDL, mmol/L *	0.89 (0.27)	0.86 (0.25)	0.79 (0.22)	0.73 (0.21)	<0.0001	<0.0001
Cholesterol, mmol/L	5.25 (0.89)	5.50 (0.87)	5.76 (0.85)	6.09 (0.92)	<0.0001	<0.0001
Triglyceride, mmol/L *	1.59 (0.79)	1.62 (0.71)	1.85 (0.85)	2.08 (0.98)	<0.0001	0.16
Fibrinogen, g/L *	2.68 (0.46)	2.69 (0.45)	2.70 (0.48)	2.73 (0.45)	0.27	0.13
CRP, mg/L *	2.83 (3.40)	2.53 (3.09)	2.86 (3.30)	3.34 (3.64)	0.001	0.10
V allele freq (95%CI)	0.17 (0.15-0.20)	0.18 (0.15-0.20)	0.18 (0.16-0.20)	0.21 (0.18-0.23)	0.14	-
AA	360 (68.2)	351 (67.6)	339 (66.2)	342 (63.2)		
AV	152 (28.8)	151 (29.1)	163 (31.8)	172 (31.8)	0.12	
VV	16 (3.0)	17 (3.3)	10 (2.0)	27 (5.00)		-
CHD (%)	40 (6.9)	41 (7.1)	54 (9.3)	50 (8.7)		
HR[1]	1.00	1.02 (0.65-1.58)	1.36 (0.90-2.06)	1.26 (0.82-1.93)	-	0.37
Adjusted HR[2]	1.00	0.88 (0.47-1.68)	1.23 (0.67-2.25)	0.96 (0.49-1.87)		0.67
CHD (%)	52 (6.7%)	65 (8.4%)	68 (8.8%)			
HR[1]	1.00	1.24 (0.86-1.79)	1.30 (0.90-1.88)		-	0.34
Adjusted HR[2]	1.00	1.34 (0.79-2.32)	1.34 (0.78-2.32)			0.48

*Natural log geometric mean. SD is approximate. [+] Suitable adjustment is for cholesterol, HDL, fibrinogen and smoking. In HR models: [1]age and practice adjusted [2]adjusted for age, practice, SBP, HDL, LDL, cholesterol, fibrinogen and smoking.

Fig 4.2.1: Kaplan-Meier survival plot of quartiles of Lp-PLA2 activity. Graph represents the probability of survival over a period of time

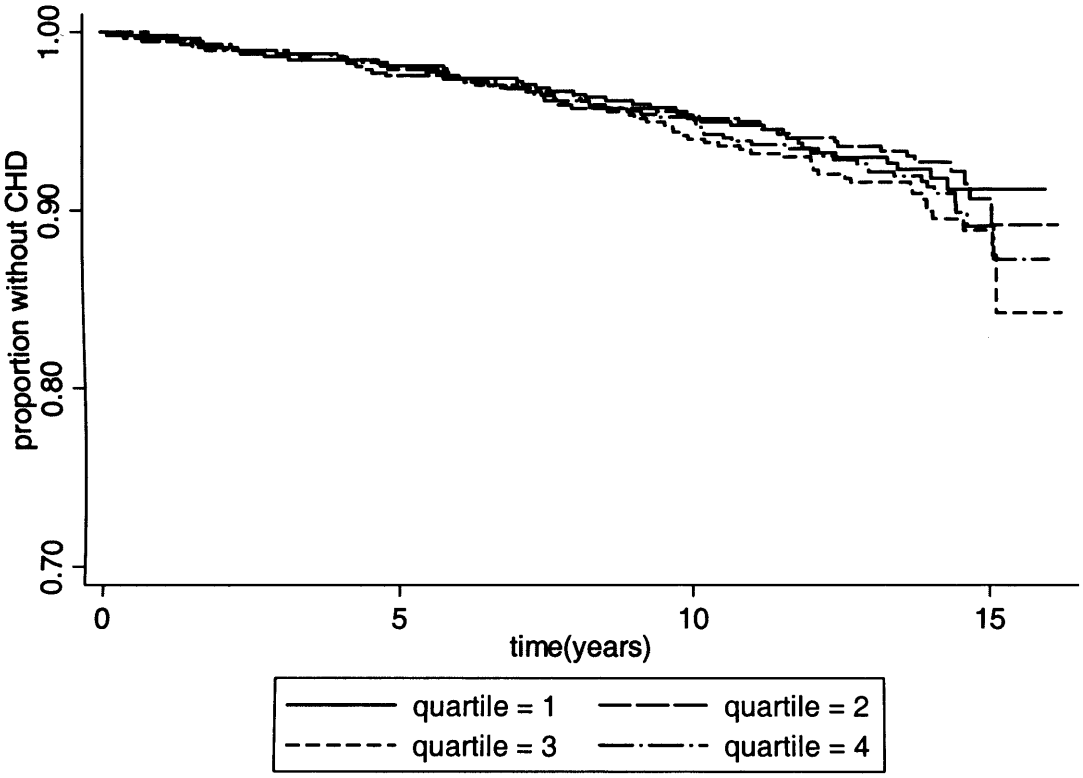


Table 4.2.4: Age of onset of CHD event by Quartile (mean± 1SD)

	Quartile1	**Quartile 2**	**Quartile 3**	**Quartile 4**	**P value**
	<39	**39-47.9**	**48-58.2**	**>58.2**	
Age at event	64.6 (5.0)	64.0 (5.6)	64.4 (5.4)	64.1 (5.2)	0.96
N	40	41	54	50	

4.2.3.4 Association of PLA2G7 A379V genotype with baseline characteristics and Lp-PLA2 activity in the entire NPHS II sample

Table 4.2.5 shows the baseline characteristics of the NPHS II study by A379V genotype. Those homozygous for the 379V allele had statistically significant higher cholesterol levels (5.99± 1.12 mmol/L) compared to AV (5.64± 1.00 mmol/L) or AA homozygous individuals (5.76± 1.01 mmol/L, p=0.0006). In addition there was a borderline trend for higher Lp-PLA2 activity (in the whole of NPHS II) in those individuals homozygous for the 379V allele (51.4± 19.9 nmol/min/ml) compared to AV (49.0± 16.5 nmol/min/ml) and AA men (48.0± 15.0 nmol/min/ml)(p=0.11, p=0.05 for trend).

Since the A379V variant is thought to act recessively (Abuzeid et al., 2003), A379 homozygous and heterozygous individuals were combined and compared against 379V homozygous individuals. Again, there was a significant association of genotype with cholesterol levels (p=0.008) and a non-significant trend (p=0.09) for higher Lp-PLA2 activity in those homozygous for the 379V allele compared to A379 carriers (Table 4.2.5).

Table 4.2.5: Baseline characteristics (mean± 1SD) by *PLA2G7* A379V genotype in NPHS II

Trait	AA N=1794	AV N=821	VV N=101	P value (ANOVA)	P value Recessive
Age, years	56.0 (3.4)	56.2 (3.5)	55.8 (3.1)	0.37	0.45
BMI kg/m^2 *	26.2 (3.4)	26.3 (3.3)	26.1 (3.4)	0.92	0.86
Systolic BP, mmHg *	136.9 (18.9)	137.5 (18.8)	136.2 (18.1)	0.69	0.66
Smoking (%)	695 (38.7%)	352 (42.9%)	47 (46.5%)	0.06	0.07
LDL, mmol/L	3.89 (0.88)	3.80 (0.87)	4.01 (0.98)	0.15	0.13
HDL, mmol/L *	0.80 (0.24)	0.80 (0.24)	0.83 (0.24)	0.64	0.40
Cholesterol, mmol/L	5.76 (1.01)	5.64 (1.00)	5.99 (1.12)	0.0006	0.008
Triglyceride, mmol/L*	1.77 (0.93)	1.82 (0.97)	1.86 (0.92)	0.39	0.17
Fibrinogen, g/L *	2.71 (0.50)	2.72 (0.55)	2.67 (0.45)	0.65	0.39
CRP, mg/L *	1.28 (1.42)	1.29 (1.44)	1.42 (1.69)	0.89	0.64
Lp-PLA2 activity, nmol/min/ml *	48.0 (15.0)	49.0 (16.5)	51.4 (19.9)	0.11 (p=0.05 trend)	0.09

*antilog of log-transformed mean. standard deviations are approximate.

4.2.3.5 Association of PLA2G7 A379V genotype with Lp-PLA2 specific activity in a subset of the NPHS II study

Collaboration with GlaxoSmithKline (GSK) led to a subset analysis of healthy individuals randomly selected by A379V genotype in the NPHS II study. The aim of this analysis was to investigate differences in Lp-PLA2 activity and mass (as measured by researchers at GSK) by genotype in order to further characterise the functional effect of this variant. Table 4.2.6 lists the baseline characteristics for the 79 A379 homozygous and 69 379V homozygous individuals chosen (blind, by our statistician) at random from the NPHS II study. There were significant differences in age (p=0.02), diastolic BP (p=0.02), cholesterol (p=0.05), and ApoB (p=0.02) measures. When Lp-PLA2 mass and activity were considered separately, there was a non-significant trend for higher Lp-PLA2 mass in the AA individuals (400.6± 112.2 ng/ml) compared to VV men (369.0± 97.9 ng/ml, p=0.08 and p=0.11 after adjustment for age practice and cholesterol levels)(Table 4.2.6). By contrast there were no significant differences in Lp-PLA2 activity between 379V (106.6± 24.6 nmol/min/ml) and A379 homozygotes (101.9± 21.4 nmol/min/ml, p=0.22 and p=0.63 after adjustment for age, practice, and cholesterol)(Table 4.2.6).

Lp-PLA2 mass and activity measures were positively correlated in both the AA (r=0.71, p<0.0001) and VV (r=0.48, p<0.0001) genotype samples, with some evidence of interaction between the AA and VV groups (Figure 4.2.2, p=0.01). The combined genotype sample also showed a high correlation between mass and activity of the enzyme (r=0.57, p<0.0001). Lp-PLA2 mass was found to be correlated with age (r=0.17, p=0.03), cholesterol (r=0.28, p<0.0001), and HDL (r=-0.28, p=0.003); while Lp-PLA2 activity was found to be correlated with cholesterol (r=0.46, p<0.0001), TG (r=0.31, p<0.0001) and HDL (r=-0.46, p<0.0001). After adjustment for age, practice, cholesterol and HDL the correlation of Lp-PLA2 activity and mass was still significant in AA379 men (r=0.57, p<0.0001) and the sample as a whole (r=0.45, p<0.0001); but only borderline significant in those 379VV men (r=0.28, p=0.09). There was no evidence of interaction between the genotype groups (p=0.12).

Specific activity corrects for the mass of enzyme present in the tested sample, and would be a better measure to accurately define differences in Lp-PLA2 activity by genotype. Lp-PLA2 mean activity (nmol/min/ml) as measured by GSK was divided by Lp-PLA2 mass (ng/ml) in the 148 individuals sampled from the NPHS II study. The specific activity measure (nmol min^{-1} ng^{-1} also termed Units per ng, U/ng) was found to not be correlated with any of the baseline characteristics of the study. When genotypes

were considered separately, specific activity was found to be significantly higher in those 379VV men (0.29± 0.08 U/ng) compared to AA homozygotes (0.25± 0.05 U/ng, p=0.001). However, after adjustment for age, diastolic BP, Cholesterol and ApoB (factors found to be significantly different by genotype in this sample), this association no longer remained significant (p=0.14)(Table 4.2.7).

Table 4.2.6: Baseline characteristics by A379V genotype of a sub-study (NPHS II) carried out in collaboration with GSK (means± 1SD)

Trait	AA N=79	VV N=69	P value
Age	57.2 (3.8)	55.8 (2.9)	0.02
BMI kg/m^2 *	26.0 (3.2)	26.0 (3.3)	0.98
SBP mmHg*	133.6 (18.2)	135.5 (17.1)	0.52
DBP mmHg	79.6 (8.4)	83.2 (9.7)	0.02
Smokers (%)	21 (26.6)	25 (36.2)	0.21
Cholesterol mmol/L	5.78 (1.03)	6.13 (1.09)	0.05
TG, mmol/L*	1.81 (0.92)	1.93 (1.05)	0.46
ApoB, mg/L*	0.85 (0.16)	0.96 (0.35)	0.02
HDL, mmol/L*	0.84 (0.26)	0.85 (0.25)	0.92
Fibrinogen, g/L *	5.54 (0.14)	5.58 (0.17)	0.06
Lp-PLA2 mass ng/ml	400.6 (112.2)	369 (97.9)	0.08
Lp-PLA2 activity nmol/min/ml	101.9 (21.4)	106.6 (24.6)	0.22

*antilog of log-transformed mean. standard deviations are approximate.

Fig 4.2.2: Scatterplot of log transformed Lp-PLA2 activity and mass. Evidence of interaction between two genotypes p=0.01.

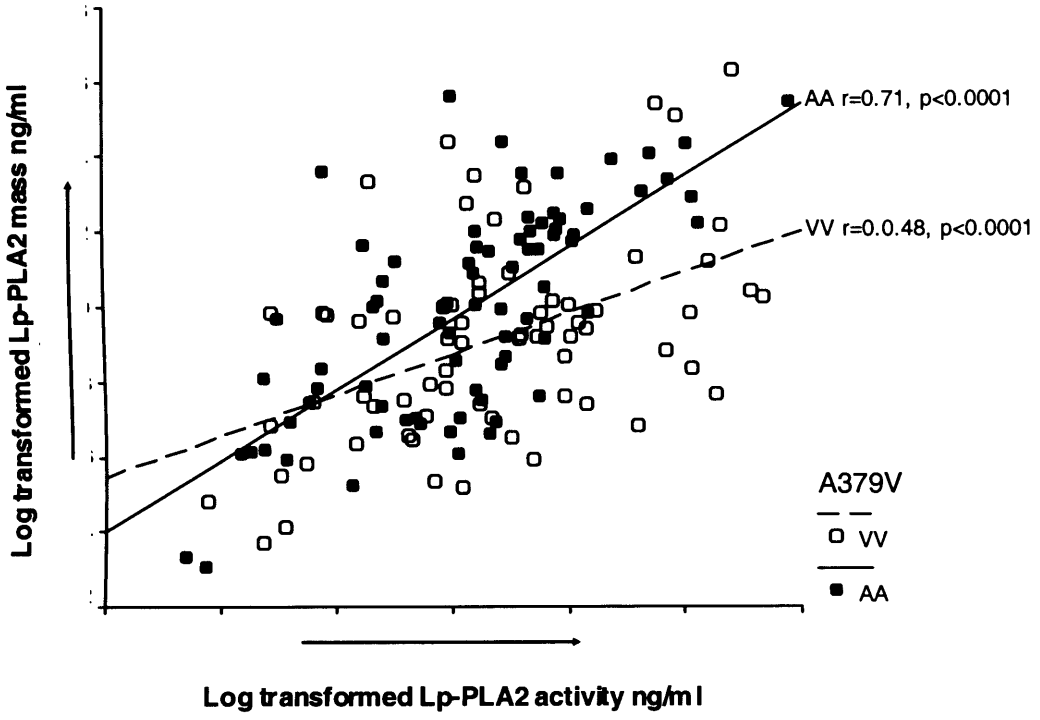


Table 4.2.7: Specific activity changes by A379V genotype

	Unadjusted U/ng	Adjusted for age, DBP, chol & ApoB
AA	0.25 (0.05)	0.27 (0.05)
VV	0.29 (0.08)	0.29 (0.07)
P value	0.001	0.14

Geometric mean (approx sd)

4.2.3.6 Association of A379V genotype with CHD risk

The association of the *PLA2G7* A379V variant with future risk of CHD was investigated in the NPHS II study as a whole, and is shown in Tables 4.2.8 and 4.2.9. Three models, adjusting for possible confounding factors were considered. In model 1 (adjusted for age and practice), there was no significant association of the A379V genotype with CHD risk when the HR was set at 1 for AA homozygous individuals (AV=1.14 95%CI 0.85-1.53, VV=0.88 95%CI 0.39-1.99, p=0.63). A similar result was also observed in model 2 adjusting for age, practice, CRP (p=0.78); and also in model 3 which adjusted for age, practice, CRP and cholesterol (p=0.66). In the recessive analysis where AA and AV men were combined, there was a suggestion of lower CHD

risk in those individuals homozygous for the 379V allele, although this did not reach statistical significance in any of the models (p>0.68)(Table 4.2.9).

When considering Kaplan-Meier survival analysis, the graphs for all three models showed no differences in survival rates (Figures 4.2.3 shows a typical pattern observed for all three models), although the right hand side of the graph does appear to show a significant difference in survival. Precision of estimates on the right hand side of Kaplan-Meier plots often suffer from small numbers (caused by drop-outs and deaths), possibly explaining the observations seen here. The step nature of the 379VV homozygous group curve is also likely to be a result of small numbers in that group.

Table 4.2.8: CHD risk by *PLA2G7* A379V genotype in the NPHS II study

A379V	Total N	No of events	Rate/ 1000person years (py)	*HR*[1] (95% CI)	HR[2] (95% CI)	HR[3] (95% CI)
AA	1794	133	7.9	1.00	1.00	1.00
AV	821	69	9.1	1.14 (0.85-1.53)	1.12 (0.81-1.57)	1.16 (0.83-1.62)
VV	101	6	6.5	0.88 (0.39-1.99)	0.96 (0.39-2.35)	0.89 (0.36-2.19)
P value				P=0.63	P=0.78	P=0.66

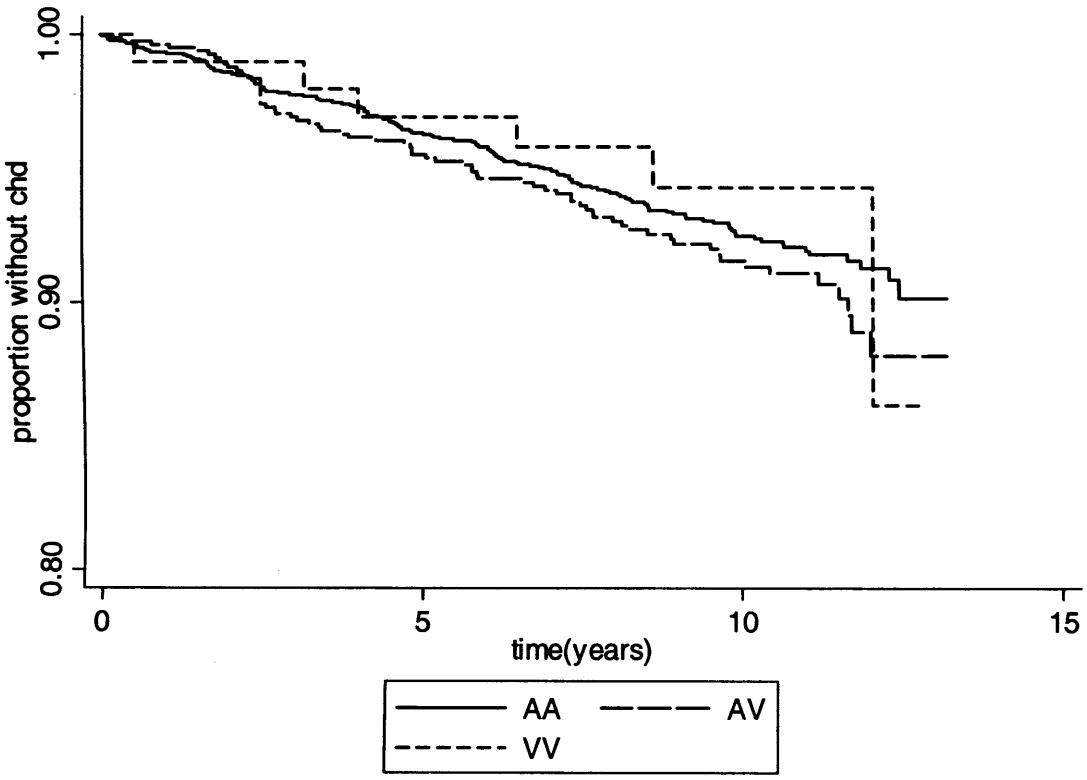
[1] age and practice adjusted
[2] adjusted for age, practice, CRP..
[3] adjusted for age, practice, cholesterol, CRP.

Table 4.2.9: CHD risk in the recessive model (combined AA and AV groups) of A379V genotype

A379V	Total N	No of events	Rate/ 1000py	*HR*[1] (95% CI)	HR[2] (95% CI)	HR[3] (95% CI)
AA/AV	2615	202	8.2	1.00	1.00	1.00
VV	101	6	6.5	0.84 (0.37-1.90)	0.92 (0.38-2.25)	0.85 (0.35-2.09)
P value				0.68	0.86	0.73

[1] age and practice adjusted
[2] adjusted for age, practice, CRP.
[3] adjusted for age, practice, cholesterol, CRP.

Fig 4.2.3: Kaplan-Meier plot of A379V genotype after adjustment for age and practice only (model 2 and model 3 curves follow a similar pattern)



4.2.4 Discussion

The aim of genotyping the NPHS II study was to confirm in a large prospective cohort, the associations previously seen between the activity altering *PLA2G7* A379V variant and CHD risk (Abuzeid et al., 2003; Ninio et al., 2004). By taking measures of Lp-PLA2 activity it would also be possible to relate activity, genotype and CHD risk in the same cohort, and determine the relationship between the Lp-PLA2 enzyme and atherosclerosis from an epidemiological stand-point.

Taking into consideration the results relating to Lp-PLA2 activity for the entire NPHS II sample, there appeared to be a significant association of quartiles of activity with LDL, HDL and total cholesterol in the NPHS II study ($p<0.01$, after adjustment). Lp-PLA2 enzyme is mainly bound to LDL and HDL (Kujiraoka et al., 2003; Noto et al., 2003), possibly explaining why these associations were found. Indeed, three residues in the primary protein sequence of the Lp-PLA2 enzyme (residue positions 115,116 and 205) are thought to be important in binding of Lp-PLA2 to ApoB in LDL (Stafforini et al., 1999), and correlations of Lp-PLA2 with lipid markers such as LDL and HDL have previously been identified (Blake et al., 2001; Blankenberg et al., 2003; Guerra et al., 1997; Packard et al., 2000; Tsimihodimos et al., 2002). Interestingly, there was also a significant association of Lp-PLA2 activity with percentage smokers. Smokers are often more susceptible to low grade inflammation (Yasue et al., 2006), and Lp-PLA2 expression is regulated by mediators of inflammation (Cao et al., 1998). This could therefore explain why there are a higher percentage of smokers in those quartiles of higher Lp-PLA2 activity. However, there was no significant difference in Lp-PLA2 activity between smokers and non-smokers in the NPHS II study, in agreement with data presented in the WOSCOPS study (Packard et al., 2000). In addition, Lp-PLA2 activity was only very weakly correlated ($r=0.04$, $p=0.04$) with the inflammatory marker CRP (there was no correlation with fibrinogen), suggesting that Lp-PLA2 activity measures were almost independent of inflammation. This result was in agreement with several other studies which have shown that mass and activity levels do not correlate with markers of inflammation (Ballantyne et al., 2004; Blankenberg et al., 2003; Packard et al., 2000; Winkler et al., 2005).

To date, a wide body of evidence from several large prospective and case-control studies has suggested that Lp-PLA2 activity and mass are strong predictors of CHD risk (Caslake et al., 2000; Blake et al., 2001; Blankenberg et al., 2003; Ballantyne et al., 2004; Winkler et al., 2005; Oei et al., 2005; Koenig et al., 2004). Contrary to these

previous studies, no association of Lp-PLA2 activity with CHD risk was found in the NPHS II study, although those individuals in the upper quartiles of Lp-PLA2 activity did appear to show a trend towards higher CHD risk (p>0.34 for both models). Lp-PLA2 activity measures at baseline were also higher in those individuals who eventually developed CHD, although this result was not statistically significant (p=0.36). A potential reason for the NPHS II study not detecting this apparently robust association may relate to the study being under-powered. The previous associations of Lp-PLA2 activity/mass with CHD risk may have been the result of the power of studies such as ARIC, WOSCOPS and AtheroGene (Ballantyne et al., 2004; Ninio et al., 2004; Packard et al., 2000). By contrast, the NPHS II study although prospective in design included a smaller number of reported cases. The lack of association seen in the NPHS II study could also relate to a different study design and end-point definition compared to other studies. However, a wide range of case-control and unselected prospective studies (with a variety of end-points) have found associations of Lp-PLA2 activity/mass with CHD risk (Ballantyne et al., 2004; Blake et al., 2001; Blankenberg et al., 2003; Caslake et al., 2000; Koenig et al., 2004; Packard et al., 2000; Winkler et al., 2005).

Unfortunately analysis of the relationship between *PLA2G7* A379V genotype, Lp-PLA2 activity and CHD risk provided contrasting evidence of the association of this enzyme with atherosclerosis. As already discussed, the 379V allele has been found to be associated with a lower CHD risk (Abuzeid et al., 2003; Ninio et al., 2004), and also altered enzyme kinetics which suggest a lower affinity for PAF substrate (Kruse et al., 2000). This in turn would suggest that the Lp-PLA2 enzyme is causally involved in CHD and represents a pro-atherogenic enzyme. However, the 379V allele was shown to be associated with a borderline significantly higher Lp-PLA2 activity (51.4± 19.9 nmol/min/ml) compared to AV (49.0± 16.5 nmol/min/ml) and AA men (48.0± 15.0 nmol/min/ml)(p=0.11, p=0.05 for trend) in the NPHS II study, replicating data from the AtheroGene study which showed a weak but statistically significant higher Lp-PLA2 activity measure in 379V allele carriers (Ninio et al., 2004). In the NPHS II study, those men homozygous for the 379V allele were also associated with higher cholesterol levels (5.99± 1.12 mmol/L) compared to AV (5.64± 1.00 mmol/L) or AA men (5.76± 1.01 mmol/L) (p=0.008, after suitable adjustment), despite the 379V allele being reported as protective against CHD.

If the 379V allele was confirmed as being associated with higher Lp-PLA2 activity (in contrast to the *in vitro* data previously published (Kruse et al., 2000)), the association of the 379V allele with higher cholesterol levels would be in accordance with the

significant positive association of Lp-PLA2 activity with cholesterol levels found in the NPHS II study. However, it has previously been shown in the HIFMECH and AtheroGene studies that the 379V allele is associated with reduced CHD risk (Abuzeid et al., 2003; Ninio et al., 2004). Therefore a higher enzyme activity associated with the 379V allele would suggest the enzyme is potentially anti-atherogenic, despite its association in the NPHS II study with higher cholesterol, a known risk factor for CHD (Huxley et al., 2002).

Supporting the association of activity and A379V genotype found in the full NPHS II sample was the subset analysis used to investigate specific activity. In this subset of randomly selected, healthy individuals there was a strong correlation of Lp-PLA2 mass to activity ($r=0.57$, $p<0.0001$) in agreement with previous data (Caslake et al., 2000). This would suggest that Lp-PLA2 contributes a large part of the PAF hydrolysing capability of plasma. Interestingly, this correlation was significantly different between the two different genotype groups and is graphically shown in Figure 4.2.2. Essentially, for the same mass of Lp-PLA2 enzyme, those individuals homozygous for the 379V allele had a higher Lp-PLA2 activity. Specific activity adjusts for the differences in enzyme mass and although mass and activity were individually not found to be significantly different by A379V genotype, randomly selected 379V homozygous individuals did have higher specific activity (0.29± 0.08 U/ng) compared to A379 homozygous men (0.25± 0.05 U/ng, $p=0.001$). Further adjustment for age, diastolic BP, cholesterol and ApoB attenuated this association, although it is unclear whether these adjustments are fully warranted since there was no significant correlation of specific activity with any other measured traits. Specific activity defines the PAF degrading activity of a fixed unit of enzymes mass and would therefore account for potential variability in enzyme mass caused by inter-group differences in LDL and HDL levels. Again, the effects seen here contradict previous assertions that the 379V Lp-PLA2 enzyme's activity is adversely altered (Kruse et al., 2000), and would support the results found in AtheroGene (Ninio et al., 2004) and to a certain extent the NPHS II sample as a whole. As a postscript to this subset analysis, the author has noticed that there were considerable differences in measured activity between the GSK method and that of Ewa Ninio's group. Lp-PLA2 activity has previously been reported in the region of 30-60 nmol/min/ml which matches the values given in the overall NPHS II analysis (Blankenberg et al., 2003; Ninio et al., 2004; Tselepis et al., 1995). Mean values obtained by GSK were elevated to nearer 100-107 nmol/min/ml. Discussions with GSK technicians suggested that the assay was functioning properly and showed a consistent inter- and intra-assay CV. The units used were reported as being identical.

As such, I have assumed that both assays are consistent and replicable and that the differences seen must be the result of different assay conditions used (Subsequent discussion with GSK technicians suggested that differences in measured activity may have been the result of the non-labelled cold PAF used in each assay).

The NPHS II study failed to confirm an association of Lp-PLA2 enzyme activity with CHD risk. There was also no association of *PLA2G7* A379V genotype with CHD risk, and Kaplan-Meier plots failed to distinguish any differences between early and late onset of CHD. Our laboratory has previously identified an association of A379V genotype with CHD risk in the HIFMECH study (which is described in more detail later in this chapter), with those individuals homozygous for the 379V allele being associated with an OR of 0.56 (0.46 after suitable adjustment)(Abuzeid et al., 2003). The HIFMECH study consists of 500 cases, with an 80% power to detect a relative risk of <0.66 when considering the reported A379V allele frequency (and also a two sided significance of 0.05). However, the NPHS II study has an 80% power to detect a relative risk of <0.57 (to a two sided significance of 0.05) when considering the observed A379V rare allele frequency (0.19). This drop in power could explain why no association of A379V genotype with CHD risk was seen in this study. In order to address the issue of study power, I decided to investigate the A379V variant and Lp-PLA2 activity in the larger EPIC-Norfolk nested case-control study of over 1000 cases matched to controls. Results from this study are discussed in detail in the next section of this chapter.

4.2.5 Summary of the NPHS II results

i) Lp-PLA2 activity was found to be positively associated with LDL, cholesterol levels, and percentage smokers, while negatively associated with HDL levels.

ii) There was no association of Lp-PLA2 activity with CHD risk, although there appeared to be a non-significant trend for higher risk of CHD in those individuals with the highest quartiles of Lp-PLA2 activity.

iii) Individuals homozygous for the 379V allele showed a borderline significant trend for higher Lp-PLA2 activity in agreement with those results from the AtheroGene study (Ninio et al., 2004). There was also an association of A379V genotype with cholesterol levels.

iv) Those individuals homozygous for the 379V allele had a significantly higher specific activity (activity/mass) compared to A379 homozygous individuals. However, this was no longer significant after adjustment.

vi) A379V genotype was found not to be associated with CHD risk, although the trend observed was similar to that found in the HIFMECH and AtheroGene study (Abuzeid et al., 2003; Ninio et al., 2004).

4.3 Association of the *PLA2G7* A379V variant with risk and Lp-PLA2 activity in the EPIC-Norfolk nested case-control study

4.3.1 Introduction

One of the more challenging aspects of direct candidate gene association studies concerns the reproducibility of significant associations (Hirschhorn and Altshuler, 2002; Ioannidis et al., 2001). Some of these concerns can be negated by using well designed and large homogeneous cohorts. The EPIC-Norfolk study provided an opportunity to further examine some of the associations found in the NPHS II study, as well as the previously reported association of *PLA2G7* A379V genotype with CHD risk (Abuzeid et al., 2003; Ninio et al., 2004). As discussed earlier in this chapter, evidence for causality of the Lp-PLA2 enzyme in the progression of atherosclerosis could be implied if there was a consistent association (in several different studies) of a functional effect genotype (in this case the A379V variant) with CHD risk.

The European Prospective Investigation into Cancer (EPIC-Norfolk) was based on recruitment from general practices in the Norfolk area of the UK, and was designed as a prospective cohort of 25,663 men and women aged 45-79 years of age recruited from 1993 onwards (Day et al., 1999). These individuals were followed for an average of 6.5 years and mortality from all causes (cardiovascular disease, cancer, etc.) was recorded. The original study was aimed at examining the prospective relationship between self-reported physical functional health and subsequent mortality. The prospective EPIC-Norfolk cohort has since been adapted to a 'nested' case-control study in order to investigate potential associations of risk markers with CHD. Nested case-control studies are at a disadvantage to prospective cohorts in that they lose the ability to study risk and intermediate phenotypes over time. However, they still remain powerful at determining relative risk between the two selected populations, and importantly, they are not subject to some aspects of selection bias that can exist with other case-control study designs (Essebag et al., 2005). With a study as large as EPIC-Norfolk, there is also the advantage of cost, since genotyping and assaying the entire sample could prove to be prohibitively expensive, with no significant advantage in terms of power (Essebag et al., 2005). The EPIC-Norfolk case-control study encompassed 1105 people in whom fatal or nonfatal CHD developed during follow-up (cases), matched with 2209 control subjects (age, sex and enrollment time). Boekholdt *et al.* previously found that both CRP and secretory PLA2 (see chapter 7) were strong predictors of CHD incidence and mortality in this study (Boekholdt et al., 2005a;

Boekholdt et al., 2005b). These associations suggested that the relationship of Lp-PLA2 activity, *PLA2G7* A379V genotype and CHD risk warranted investigation in the EPIC-Norfolk study. Analysis of EPIC-Norfolk would determine whether the previous associations of *PLA2G7* genotype and/or Lp-PLA2 activity with CHD risk seen in the HIFMECH (Abuzeid et al., 2003), AtheroGene (Ninio et al., 2004) and NPHS II studies were valid.

4.3.2 Materials and Methods

4.3.2.1 EPIC-Norfolk study

The European Prospective Investigation into Cancer and Nutrition (EPIC-Norfolk) study consisted of a prospective population study of 25,663 men and women aged between 45 and 79 years, resident in Norfolk, UK, who completed a baseline questionnaire survey and attended a clinic visit (Day et al., 1999). Participants were recruited from age–sex registers of general practices in Norfolk as part of the 10-country collaborative EPIC study designed to investigate dietary and other determinants of cancer. Additional data were obtained in EPIC-Norfolk to enable the assessment of determinants of other diseases. At the baseline survey between 1993 and 1997, participants completed a detailed health and lifestyle questionnaire. All individuals were flagged for death certification at the UK Office of National Statistics, with vital status ascertained for the entire cohort. In addition, participants admitted to hospital were identified using their unique National Health Service number by data linkage with ENCORE (East Norfolk Health Authority database), which identifies all hospital contacts throughout England and Wales for Norfolk residents. Participants were identified as having CHD during follow-up if they had a hospital admission and/or died with CHD as the underlying cause. CHD was defined as codes 410 to 414 according to the International Classification of Diseases 9th revision. These codes encompass the clinical spectrum of CHD, i.e., unstable angina, stable angina, and myocardial infarction. The study was approved by the Norwich District Health Authority Ethics Committee and all participants gave signed informed consent.

Clinical measurements

Along with height and weight measurements, blood pressure was measured using an Accutorr Sphygmomanometer after the participant had been seated for 3 minutes. Two measurements of blood pressure were taken and the mean of the readings was used in analysis (Day et al., 1999; Myint et al., 2005). Non-fasting blood samples were taken by venepuncture into plain and citrate bottles. Blood samples were processed for assay at the Department of Clinical Biochemistry, University of Cambridge, or stored at –80°C. Serum concentrations of secretory PLA2 were measured with a sandwich-type enzyme-linked immunosorbent assay as previously described (Wolbink et al., 1995). Plasma concentrations of CRP were measured with a sandwich-type enzyme-linked immunosorbent assay as previously described (Bruins et al., 1997). Results were related to a standard consisting of commercially available CRP (Behringwerke AG, Marburg, Germany). Serum levels of total cholesterol, HDL-C, and triglycerides were

measured in fresh plasma samples with the RA 1000 (Bayer Diagnostics), and LDL-C levels were calculated with the Friedewald formula (Friedewald et al., 1972).

EPIC-Norfolk nested case-control study

A similar designed EPIC-Norfolk nested case-control study has been described where 755 apparently healthy individuals ultimately developed fatal or non-fatal CHD during follow-up (Boekholdt et al., 2004). However, an extended follow-up has resulted in the identification of additional CHD cases, allowing the present study to be larger (Boekholdt et al., 2005b; Boekholdt et al., 2005a). All individuals who reported a history of heart attack or stroke at the baseline clinic visit were excluded. Cases were 1043 individuals in whom a fatal or nonfatal CHD developed during follow-up until November 2003. Controls were study participants who remained free of any cardiovascular disease during follow-up. At least two controls were matched up to each case by sex, age (within 5 years), and time of enrolment (within 3 months). A total of 2094 controls could be matched to cases.

4.3.2.2 Lp-PLA2 activity measures

Activity measures for the entire EPIC-Norfolk sample were kindly assayed by Ewa Ninio's group based in France (INSERM-Paris), using the 96-well plate format, trichloroacetic acid precipitation procedure (Blankenberg et al., 2003; Tselepis et al., 1995). A full description of this protocol can be found in section 4.2.2.3 of this chapter.

4.3.2.3 *PLA2G7* A379V genotyping

Genotyping of the case-control EPIC-Norfolk study was completed using the Taqman® assay. Details of the Assay design, reagents and cycling conditions can be found in chapter 2, section 2.2.6. Table 2.3 also lists the probes and oligonucleotides used.

4.3.3.4 Statistical analysis

Statistical analysis was carried out by Dr Manjinder Sandhu at the Institute of Public Health, Cambridge University using SPSS v12.0.1. Baseline characteristics were compared between cases and controls taking into account the matching between them. A mixed effect model was used for continuous variables and conditional logistic regression was used for categorical variables. TG, CRP, secretory PLA2, and Lp-PLA2 measures have a skewed distribution, therefore values were log-transformed before being used as continuous variables in statistical analysis. Tables for the EPIC-Norfolk study differ from those of NPHS II, UDACS, HIFMECH and BH2 in that un-transformed values for these measures are shown along with their relevant interquartile ranges

(IQR). Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) as an estimate of the relative risk of incident CHD were calculated using conditional logistic regression analysis taking into account matching for sex, age and time of enrolment (model 1). Odds ratios were calculated for quartiles of the Lp-PLA2 activity distribution among controls at baseline. The lowest Lp-PLA2 activity quartile was used as the reference category, and in genotype analysis the common homozygous A379 group was set as the reference. In addition to those adjustments made in model 1, model 2 adjusted for BMI, smoking, diabetes and systolic BP; and model 3 additionally adjusted for total cholesterol and HDL. For all analyses, a p-value <0.05 was considered to indicate statistical significance.

4.3.3 Results

4.3.3.1 Baseline characteristics of the EPIC-Norfolk study by CHD status

Table 4.3.1 shows the baseline characteristics of the EPIC-Norfolk case-control study by CHD status. Those individuals who had experienced a CHD event were more likely to be smokers and suffering from diabetes; as well as having a higher BMI, systolic BP, cholesterol, TG, CRP, and sPLA2 measures ($p<0.0001$ for each). They also showed lower levels of HDL ($p<0.0001$). Interestingly mean Lp-PLA2 activity was elevated in the CHD cases (51.6 (42.3-62.2) nmol/min/ml) compared to controls (49.2 (39.7-59.9) nmol/min/ml, $p<0.0001$), and this association would be tested further in section 4.3.3.3.

4.3.3.2 Association of Lp-PLA2 activity with risk factors

Tables 4.2.3 A/B show the association of risk factors in the EPIC-Norfolk study with quartiles of Lp-PLA2 activity in both male and female controls separately. In agreement with results from the NPHS II study, there were significant associations (in both male and female individuals) of higher Lp-PLA2 activity with higher TG ($p<0.0001$), LDL ($p<0.0001$) and cholesterol ($p<0.0001$) levels; and lower levels of HDL ($p<0.0001$). Those men in the higher quartiles of Lp-PLA2 activity tended to be significantly older ($p=0.03$), had a higher BMI ($p=0.002$) and a borderline significantly higher systolic BP ($p=0.05$). In women, those in the higher quartiles of Lp-PLA2 activity were also significantly older ($p<0.0001$) and were associated with a significantly higher systolic BP ($p=0.01$).

4.3.3.3 Association of Lp-PLA2 activity with CHD risk

The association of Lp-PLA2 activity with risk of CHD was investigated in the EPIC-Norfolk study and is shown in Table 4.3.3 and Figure 4.3.1. Quartiles of Lp-PLA2 activity showed a strong association with CHD risk in model 1 analysis, with all quartiles associated with a higher OR when compared to the lowest Lp-PLA2 activity quartile set as the reference ($p<0.0001$). This association remained highly significant in model 2 adjusting for BMI, smoking status, diabetes, and systolic BP ($p<0.0001$). Unfortunately, model 3 which includes further adjustment for cholesterol and HDL led to the association no longer remaining significant ($p=0.45$). Using a log likelihood test to compare Lp-PLA2 as a non-categorical variable (i.e. quartiles), Lp-PLA2 activity did not predict risk of CHD events after adjustment for those factors in model 3 ($p=0.20$). In addition, using a continuous variable for Lp-PLA2 (log transformed) instead of a categorical variable and model 3, it was found that there was no interaction with sex on this scale ($p= 0.90$); and that there was no further detectable interaction with age, total

cholesterol, HDL or CRP (all p> 0.70). This would suggest that the adjustments made were valid.

Table 4.3.1: Baseline characteristics of CHD cases and controls, EPIC-Norfolk.

Trait		Controls n = 2094	Cases n = 1043	P-value
Age, years		65 ± 8	65 ± 8	Matched
Men (%)		1311 (63)	669 (64)	Matched
Smoking:	**Never (%)**	850 (41)	344 (33)	
	Previous (%)	1073 (51)	539 (52)	< 0.0001
	Current (%)	171 (8)	160 (15)	
Body mass index, kg/m^2		26.2 ± 3.4	27.2 ± 3.9	< 0.0001
Diabetes (%)		35 (2)	66 (6)	< 0.0001
Systolic blood pressure, mmHg		139 ± 18	144 ± 19	< 0.0001
Total cholesterol, mmol/l		6.2 ± 1.1	6.5 ± 1.2	< 0.0001
LDL cholesterol, mmol/l		4.1 ± 1.0	4.3 ± 1.0	< 0.0001
HDL cholesterol, mmol/l		1.37 ± 0.40	1.27 ± 0.37	< 0.0001
Triglycerides, mmol/l*		1.6 (1.1–2.2)	1.8 (1.3–2.6)	< 0.0001
C-reactive protein, mg/l*		1.5 (0.7–3.1)	2.2 (1.0–4.9)	< 0.0001
sPLA2, ng/ml*		8.3 (5.8–12.7)	9.3 (6.3–14.9)	< 0.0001
Lp-PLA2 activity nmol/min/ml*		49.2 (39.7–59.9)	51.6 (42.3–62.2)	< 0.0001

Data are means and standard deviations, unless otherwise stated
* Data are medians (interquartile range)
Numbers vary due to missing values for CRP and sPLA2

Table 4.3.2 A: Association between Lp-PLA2 and risk factors for CHD in controls at baseline (men).

Quartiles of Lp-PLA2		**1**	**2**	**3**	**4**	**P-value**
		n = 328	**n = 328**	**n = 328**	**n = 327**	
Lp-PLA2 activity range		< 42.1	42.1–51.3	51.4–62.3	> 62.3	
Age, year		65 ± 8	66 ± 7	68 ± 6	68 ± 6	0.03
Smoking:	**Never (%)**	104 (32)	109 (33)	97 (29)	108 (33)	
	Previous (%)	200 (61)	190 (58)	209 (64)	184 (56)	0.40
	Current (%)	24 (7)	29 (9)	22 (7)	35 (11)	
Body mass index, kg/m^2		26.0 ± 3.2	26.1 ± 3.0	26.3 ± 2.9	26.7 ± 3.3	0.002
Diabetes (%)		9 (3)	7 (2)	6 (2)	6 (2)	0.72
Systolic blood pressure,mmHg		138 ± 17	139 ± 18	142 ± 18	139 ± 17	0.05
Total cholesterol, mmol/l		5.5 ± 0.9	5.9 ± 0.9	6.2 ± 1.0	6.5 ± 1.0	< 0.0001
LDL cholesterol, mmol/l		3.4 ± 0.8	3.8 ± 0.8	4.1 ± 0.8	4.4 ± 1.0	< 0.0001
HDL cholesterol, mmol/l		1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	< 0.0001
Triglycerides, mmol/l*		1.5 (1.0–2.1)	1.6 (1.2–2.2)	1.8 (1.3–2.3)	1.9 (1.3–2.5)	< 0.0001
C-reactive protein, mg/l*		1.2 (0.5–2.6)	1.4 (0.7–3.0)	1.5 (0.7–3.0)	1.5 (0.8–3.0)	0.10
sPLA2, ng/ml*		6.8 (5.2–10.0)	7.5 (5.2–11.4)	7.5 (5.5–10.6)	7.2 (5.3–10.5)	0.57

Data are means and standard deviations, unless otherwise stated

* Data are medians (interquartile range) and P-values based on linear regression using log transformation

Numbers vary due to missing values for CRP, sPLA2

Table 4.3.2 B: Association between Lp-PLA2 and risk factors for CAD in controls at baseline (women).

Quartiles of Lp-PLA2		**1**	**2**	**3**	**4**	**P-value**
		n = 197	**n = 195**	**n = 196**	**n = 195**	
Lp-PLA2 activity range		< 36.2	36.2–45.5	45.6–55.3	> 55.3	
Age, year		64 ± 9	64 ± 8	65 ± 8	65 ± 7	<0.0001
Smoking:	**Never (%)**	108 (55)	114 (58)	113 (58)	97 (50)	
	Previous (%)	73 (37)	62 (32)	75 (38)	80 (41)	0.24
	Current (%)	16 (8)	19 (10)	8 (4)	18 (9)	
Body mass index, kg/m^2		25.7 ± 3.9	26.4 ± 4.3	26.1 ± 4.0	26.3 ± 3.6	0.29
Diabetes (%)		2 (1)	4 (2)	1 (0.5)	0 (0)	0.25
Systolic blood pressure mmHg		135 ± 20	139 ± 18	140 ± 18	139 ± 18	0.01
Total cholesterol, mmol/l		5.9 ± 1.0	6.3 ± 0.9	6.7 ± 0.9	7.4 ± 1.3	< 0.0001
LDL cholesterol, mmol/l		3.6 ± 0.9	4.0 ± 0.8	4.4 ± 0.8	5.0 ± 1.1	< 0.0001
HDL cholesterol, mmol/l		1.7 ± 0.4	1.6 ± 0.4	1.6 ± 0.4	1.5 ± 0.4	< 0.0001
Triglycerides, mmol/l*		1.2 (1.0–1.7)	1.4 (1.1–2.0)	1.6 (1.2–2.1)	1.7 (1.1–2.5)	< 0.0001
C-reactive protein, mg/l*		1.6 (0.7–3.6)	1.7 (0.7–4.1)	1.5 (0.7–3.1)	1.6 (0.8–3.4)	0.74
sPLA2, ng/ml*		9.7 (7.0–17.5)	11.2 (7.4–17.1)	9.8 (7.1–15.0)	11.3 (7.6–15.8)	0.52

Data are means and standard deviations, unless otherwise stated
* Data are medians (interquartile range) and P-values based on linear regression using log transformation
Numbers vary due to missing values for CRP, sPLA2

Table 4.3.3: Association between Lp-PLA2 and risk of CHD, EPIC-Norfolk, 1993–2003

Lp-PLA2 activity	*Percentile*	Cases (%)	Controls (%)	Model 1: adjusted odds ratio (95% CI) *	Model 2: adjusted odds ratio (95% CI) **	Model 3: Adjusted odds ratio (95% CI) ***
< 40.7	**< 25th**	214 (21)	573 (27)	1.00	1.00	1.00
40.7–49.9	**25th–49th**	251 (24)	531 (25)	1.26 (1.02–1.57)	1.23 (0.98–1.54)	1.11 (0.88–1.40)
50.0–60.7	**50th–75th**	291 (28)	493 (24)	1.57 (1.27–1.95)	1.51 (1.21–1.89)	1.26 (1.00–1.59)
> 60.7	**> 75th**	287 (27)	497 (24)	1.54 (1.24–1.91)	1.43 (1.14–1.79)	1.06 (0.83–1.37)
P-trend (1 df)				**< 0.0001**	**< 0.0001**	**0.45†**

* Model 1: Adjusted for age (continuous), sex and enrolment date

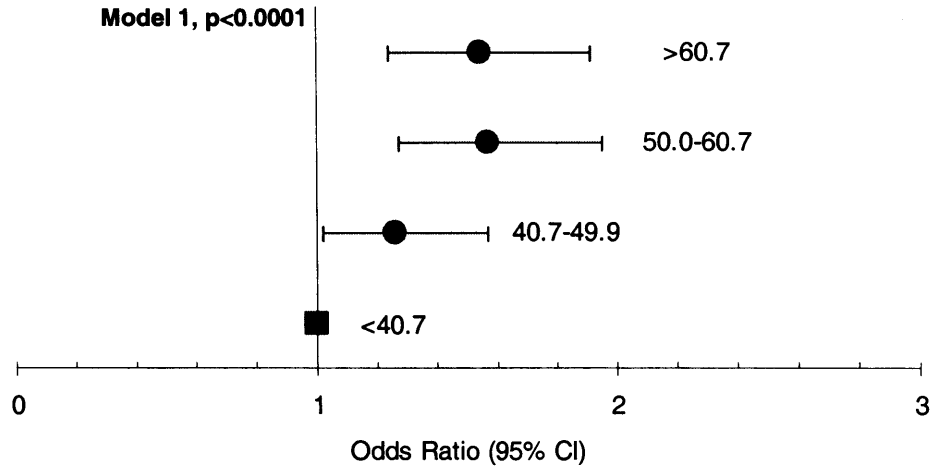
** Model 2: Adjusted for age (continuous), sex and enrolment date, bmi (continuous), smoking (categorical), diabetes and systolic blood pressure (continuous)

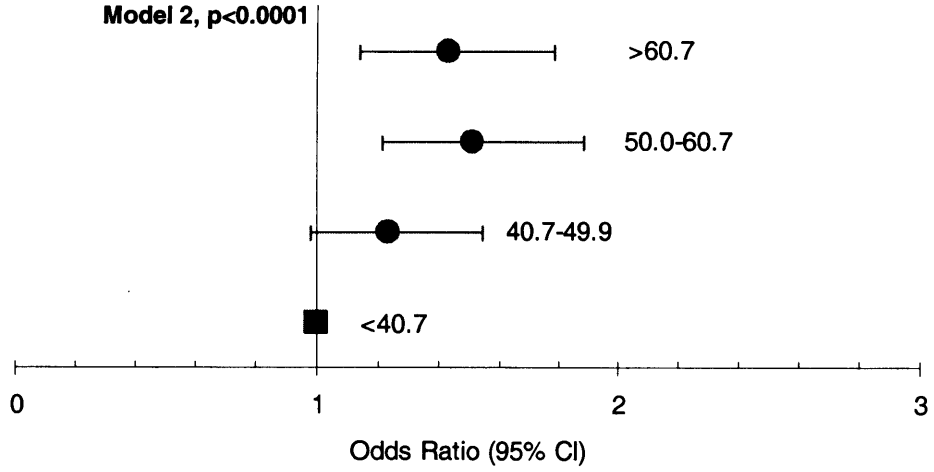
*** Model 3: Adjusted for age (continuous), sex and enrolment date, bmi (continuous), smoking (categorical), diabetes, systolic blood pressure (continuous), total cholesterol (continuous) and HDL (continuous)

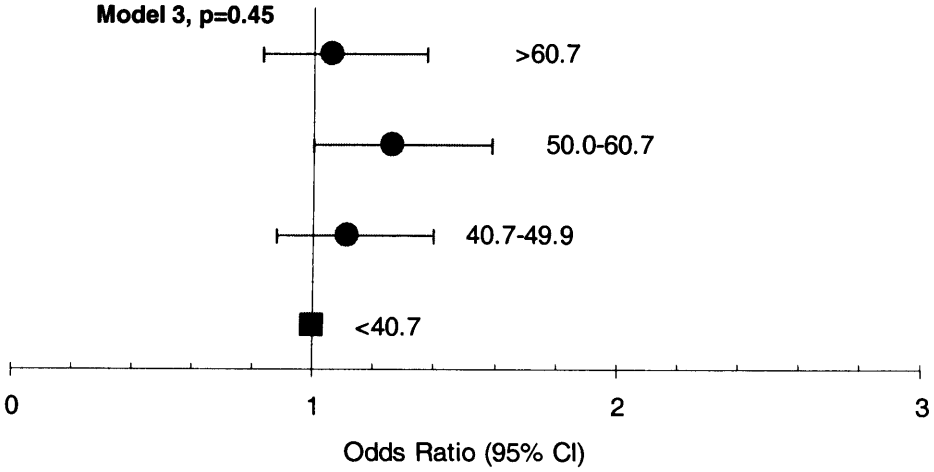
† p = 0.20 for a log likelihood ratio test comparing model 3 with and without a categorical variable for Lp-PLA2 (quartiles), suggesting that Lp-PLA2 does not predict risk of CHD after adjusting for risk factors for CHD.

Using a continuous variable for Lp-PLA2 (log transformed) instead of a categorical variable and model 3, we found no sex interaction on this scale; p = 0.90. We found no detectable interaction with age, total cholesterol, HDL or CRP (all P > 0.70)

Fig 4.3.1: Graphical representation of the ORs for each quartile of Lp-PLA2 activity (nmol/min/ml). See table 4.3.3 for details of each model.







4.3.3.4 Association of *PLA2G7* A379V genotype with risk factors

Table 4.3.4 lists the baseline characteristics in the control subjects of the EPIC-Norfolk study by *PLA2G7* A379V genotype. There was no significant association of the A379V genotype with any of the measured characteristics apart from Lp-PLA2 activity. Individuals homozygous for the 379V allele were associated with a weak but significantly higher Lp-PLA2 activity (53.6 (40.7-63.2) nmol/min/ml) compared to AV (49.7 (41.1-62.5) nmol/min/ml) and AA (48.9 (39.5-58.7) nmol/min/ml) homozygous individuals (p=0.03). As discussed earlier, the A379V variant is thought to act recessively (Abuzeid et al., 2003). When A379 homozygous and heterozygous individuals were combined, the previous association with Lp-PLA2 activity was no longer statistically significant (p=0.20), although there did appear to be a borderline significantly lower HDL level in those homozygous for the 379V allele (p=0.04).

4.3.3.5 Association of *PLA2G7* A379V genotype with CHD risk

When considering the association of A379V genotype with CHD risk, contrary to other associations that have found a significantly lower CHD risk associated with 379VV homozygous individuals, the EPIC-Norfolk study showed no association of the A379V genotype with CHD risk in any of the models tested (p>0.90). When considering the recessive model of combined AA and AV genotypes, there was a borderline significantly higher CHD risk in those individuals homozygous for the 379V allele (model 1, VV=1.44 95%CI 0.99-2.12, p=0.06)(Table 4.3.5 and Fig. 4.3.2). Interestingly, when the model was over-adjusted by including Lp-PLA2 activity, the borderline association remained similar in these models (model 2 and 3, p>0.07). This would suggest that the association of the A379V variant with CHD risk, was independent of Lp-PLA2 activity measures (Table 4.3.5 and Fig. 4.3.2). In additional analyses, ORs were not materially different in models adjusting for HDL, LDL, CRP and TG levels (as log transformed continuous variables).

Table 4.3.4: Association between A379V genotype and risk factors for CHD in controls

A379V genetic variant	AA	AV	VV	P value (additive)	P value (recessive)
	n = 1158	n = 581	n = 66		
Age, year	65 ± 8	66 ± 8	66 ± 8	0.1	0.6
Men (%)	749 (65)	371 (64)	49 (74)	0.5	0.1
Smoking: Never (%)	459 (40)	228 (39)	26 (39)		
Previous (%)	598 (51)	311 (54)	35 (53)	0.8*	0.9
Current (%)	101 (9)	42 (7)	5 (8)		
Body mass index, kg/m^2	26.2 ± 3.4	26.3 ± 3.6	26.0 ± 3.2	0.9	0.6
Diabetes (%)	21 (2)	9 (2)	1 (2)	0.7	0.9
Systolic blood pressure, mmHg	139 ± 17	140 ± 18	139 ± 15	0.3	0.9
Total cholesterol, mmol/l	6.2 ± 1.1	6.2 ± 1.1	6.2 ± 1.3	0.6	0.8
LDL cholesterol, mmol/l	4.1 ± 1.0	4.0 ± 1.0	4.1 ± 1.1	0.5	0.9
HDL cholesterol, mmol/l	1.36 ± 0.40	1.40 ± 0.41	1.27 ± 0.37	0.7	0.03
Triglycerides, mmol/l*	1.6 (1.1–2.2)	1.5 (1.1–2.2)	1.8 (1.3–2.5)	0.9	0.06
C-reactive protein, mg/l*	1.4 (0.7–3.0)	1.5 (0.7–3.1)	1.8 (0.6–3.2)	0.9	0.7
sPLA2, ng/ml*	8.2 (5.8–12.4)	7.8 (5.8–11.7)	8.0 (5.9–13.3)	0.3	0.9
Lp_PLA2 activity, nmol/min/ml*	48.9 (39.5–58.7)	49.7 (41.1–62.5)	53.6 (40.7–63.2)	0.03	0.2

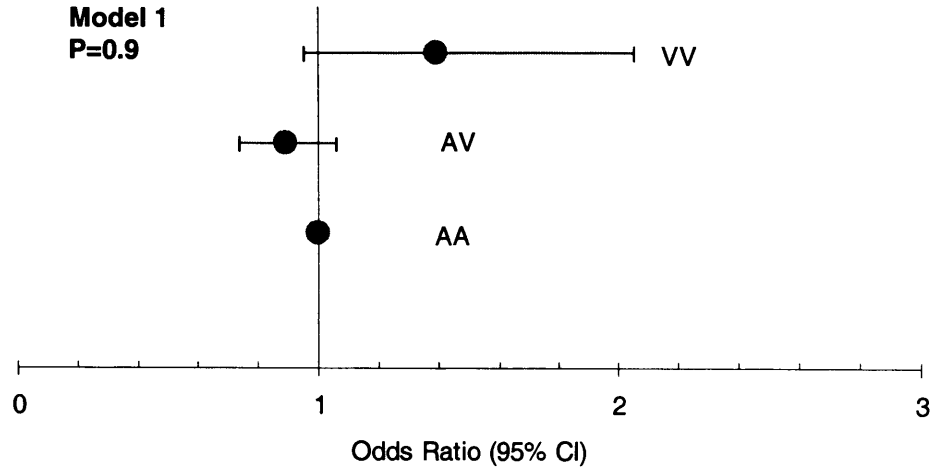
The variant was in Hardy–Weinberg equilibrium (P = 0.50)
* Chi-squared test for heterogeneity

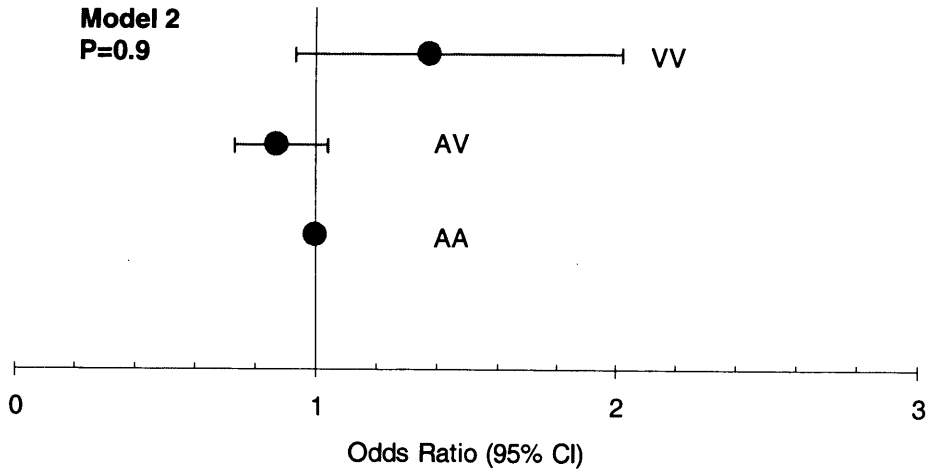
Table 4.3.5: Association between Lp-PLA2 A379V genetic variant and risk of CHD, EPIC-Norfolk, 1993–2003

A379V genetic variant	*Genotype*	Controls (%)	Cases (%)	Model 1: adjusted odds ratio (95% CI)	Model 2: adjusted odds ratio (95% CI)	Model 3: adjusted odds ratio (95% CI)
	AA	1247 (64)	646 (64)	1.00	1.00	1.00
	AV	618 (32)	298 (30)	0.89 (0.74-1.06)	0.87 (0.73–1.04)	0.89 (0.74-1.06)
	VV	71 (4)	56 (6)	1.39 (0.95-2.05)	1.37 (0.93–2.02)	1.33 (0.90-1.96)
P for trend				**0.9**	**0.9**	**0.9**
Recessive				1.44 (0.99–2.12)	1.43 (0.98–2.10)	1.38 (0.93–2.03)
P value				**0.06**	**0.07**	**0.15**

CI, confidence interval
Model 1: adjusted for age and sex, and enrolment date
Model 2: adjusted for age and sex, enrolment date and log Lp-PLA2 levels
Model 3: adjusted for age and sex, enrolment date, log Lp-PLA2, HDL and LDL

Fig 4.3.2: Graphical representation of the ORs for A379V genotype. See table 4.3.5 for details of each model. Third Graph shows the AA/AV combined genotype analysis and the OR for the three models.





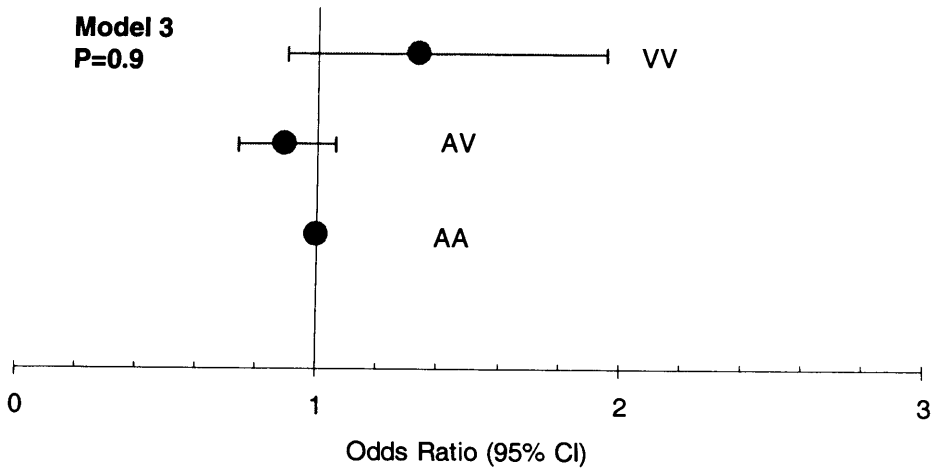
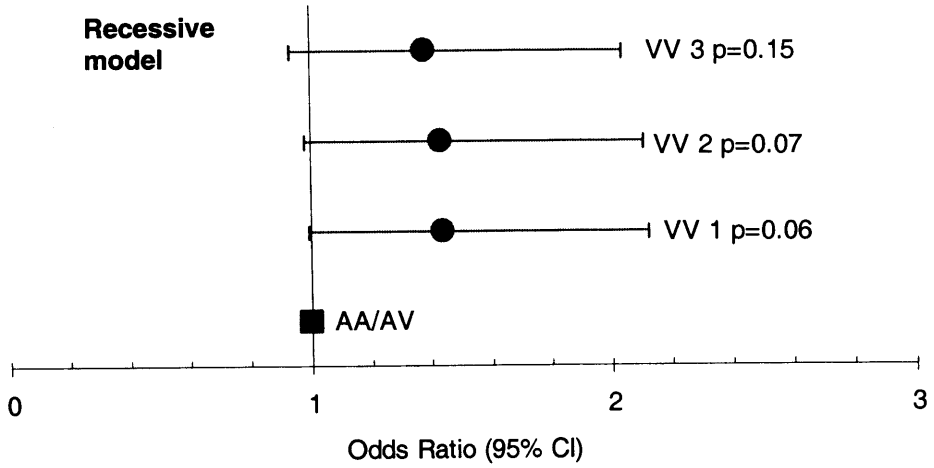


Fig. 4.3.2: continued



4.3.4 Discussion

Investigating *PLA2G7* A379V genotype, Lp-PLA2 activity and CHD risk in the NPHS II study did not provide conclusive evidence of a causal relationship of the Lp-PLA2 enzyme with atherosclerosis. One of the reasons for this could relate to the NPHS II study being under-powered to investigate these associations. By contrast the EPIC-Norfolk study provided a more powerful study with an 80% power to detect a relative risk of <0.76 and >1.29 for the A379V genotype (based on the observed rare allele frequency for the EPIC-Norfolk cohort of 0.20 and a 0.05 two sided level of significance).

The baseline characteristics of the EPIC-Norfolk study by quartiles of Lp-PLA2 activity supported those results found previously in the NPHS II study, namely the association of Lp-PLA2 activity with markers of lipid metabolism. However, in addition to these robust associations it appeared that those individuals in the higher quartiles of Lp-PLA2 activity were older, had a higher systolic BP and in the case of the men, had higher BMI scores. Other large scale studies investigating Lp-PLA2 have failed to find an association or correlation with blood pressure or BMI (Koenig et al., 2004; Winkler et al., 2005), suggesting that the results seen here may be independent of Lp-PLA2 and could relate to the over representation of older individuals in the highest quartiles of Lp-PLA2 activity.

In contrast to the findings of the NPHS II study, mean Lp-PLA2 activity was found to be significantly higher in CHD cases (51.6 (42.3-62.2) nmol/min/ml) compared to controls (49.2 (39.7-59.9) nmol/min/ml) in the EPIC-Norfolk study ($p<0.0001$). When considering quartiles of Lp-PLA2 activity, this association remained, with those individuals in the highest quartiles associated with a significantly higher risk of CHD ($p<0.0001$). Adjustment for age, sex and enrolment date, BMI, smoking, diabetes and systolic BP did not significantly alter this result ($p<0.0001$), but further adjustment for cholesterol and HDL led to the removal of this significant association ($p=0.45$). The adjustment for cholesterol and subsequent loss of association seen in EPIC-Norfolk replicates some of the previously published data that has investigated the relationship of Lp-PLA2 with CHD risk (Ballantyne et al., 2004; Brilakis et al., 2005). In this case, the marking of disease by Lp-PLA2 could have been the result of confounding, where Lp-PLA2 marks the effects of another functionally important factor (in this case cholesterol and LDL). However, results from the Rotterdam, Ludwigshafen, and

WOSCOPS studies have suggested that although the association of Lp-PLA2 activity/mass with CHD risk was attenuated after adjustment for non-HDL cholesterol levels, it still remained statistically significant (Ballantyne et al., 2004; Oei et al., 2005; Packard et al., 2000; Winkler et al., 2005). Despite the loss of an association after adjustment for cholesterol, the results from EPIC-Norfolk do support results from several different experimental designs that have successfully identified Lp-PLA2 activity and mass as markers of atherosclerosis (Caslake et al., 2000; Blake et al., 2001; Blankenberg et al., 2003; Ballantyne et al., 2004; Winkler et al., 2005; Oei et al., 2005; Packard et al., 2000; Iribarren et al., 2005; Koenig et al., 2004).

When considering *PLA2G7* A379V genotype and Lp-PLA2 activity, a familiar pattern to that of the NPHS II and AtheroGene studies (Ninio et al., 2004) was observed. The 379V allele was found to be associated with a higher Lp-PLA2 activity ($p=0.03$), and although the difference was small, it was statistically significant. The consistent association of the 379V allele with higher Lp-PLA2 activity (measured using this immuno-assay method) raises intriguing questions about Lp-PLA2 enzyme's role in atherosclerosis. Previous to this thesis, only the HIFMECH and AtheroGene studies had investigated the association of A379V genotype with CHD risk, with both studies suggesting that the 379V allele was protective (Abuzeid et al., 2003; Ninio et al., 2004). As already discussed in the previous section, if the 379V allele was found to be associated with a significantly higher Lp-PLA2 activity but also a reduced risk of CHD, it would suggest that the Lp-PLA2 enzyme is primarily anti-atherogenic in human subjects. However, there are still several issues that have not been addressed by the published work or work presented in this thesis. Firstly, Lp-PLA2 activity and mass measures have been generally shown to be higher in those individuals with CHD [reviewed in (Sudhir, 2005)] appearing to contradict the observations relating A379V genotype to enzyme activity and CHD risk. An explanation of this could be that higher Lp-PLA2 levels in those individuals at risk of CHD could represent an adaptive mechanism to disease (reverse-causation).

There are also issues surrounding the suitability of using a PAF devised activity assay. Some researchers have questioned whether the Lp-PLA2 enzyme's primary role *in vivo* is the hydrolysis of PAF, with one particular study showing that specific inhibitors of Lp-PLA2 do not affect plasma concentrations of PAF (Zalewski and Macphee, 2005). Intravenous administration of recombinant human Lp-PLA2 was also found not to affect PAF-mediated responses in patients with asthma or those with septic shock (Henig et

al., 2000; Opal et al., 2004). Much of the pro-atherogenic action of Lp-PLA2 relates to the formation of downstream inflammatory mediators from oxidised phospholipids (Zalewski and Macphee, 2005). The Lp-PLA2 activity assay used in this thesis measures the specific ability of the Lp-PLA2 enzyme to break down radio-labelled PAF. While it is unlikely that contamination from other enzymes such as paraoxonase or other PLA2 family members are having a significant effect on these measures, it does raise the question of whether measuring the rate of PAF hydrolysis *in vitro* and inferring enzyme activity from that, is directly comparable to the *in vivo* hydrolysis of a range of phospholipid substrates. In addition to these problems, results from the AtheroGene, NPHS II, and EPIC-Norfolk studies imply that the Lp-PLA2 altering effects of the A379V genotype account for a comparatively small variability of plasma Lp-PLA2 activity, and in the case of the AtheroGene study there was the suggestion that this difference in activity by A379V genotype was unlikely to fully explain the effect seen on CHD risk (Ninio et al., 2004).

Genotype is not subject to confounding (a particular problem when investigating Lp-PLA2) since it is determined at conception by the random inheritance of one of each parental allele (Minelli et al., 2004; Thomas and Conti, 2004), therefore the A379V variant was seen as a useful tool for overcoming the potential confounding seen in analysis of Lp-PLA2 activity/mass measures in relation to CHD risk. Unfortunately, the EPIC-Norfolk study failed to find an association of A379V genotype with CHD risk. If anything, there appeared to be a trend for the 379V homozygous individuals to be at a higher risk of CHD (contradicting previous associations (Abuzeid et al., 2003; Ninio et al., 2004)), and in the recessive model of combined AA and AV genotypes this reached borderline significance (model 1 p=0.06, model 2 p=0.07, and model 3 p=0.15). The EPIC-Norfolk study represents one of the largest studies to date that has investigated the association of *PLA2G7* A379V genotype with CHD risk, and as such, the lack of a significant association with CHD risk in this study cannot be explained by any loss in power. There is the possibility that the A379V polymorphism is not a truly functional variant, and that other SNPs in LD with the A379V variant could be more strongly affecting enzyme activity or function. However, evidence from Ninio *et al.* has shown that both in single locus and haplotype analyses the A379V variant was the main determinant of CHD risk in the AtheroGene study (Ninio et al., 2004). Of course this does not rule out the possibility that other, as yet unidentified SNPs could also be having an effect on enzyme function, thereby diluting the effect of the variant under investigation (Hirschhorn and Altshuler, 2002; Ninio et al., 2004; Suh and Vijg, 2005).

Interestingly, when model 2 and model 3 were over-adjusted by the addition of Lp-PLA2 activity, the non-significant trends with regards to CHD risk appeared not to alter (one would expect the any association to disappear). Statistically this would suggest that the association with CHD risk is independent of the Lp-PLA2 activity measure. One could infer from this that the A379V variant is either not functional, or that the Lp-PLA2 activity measure does not accurately represent the functional effect of this SNP.

Apart from these concerns, there also remains a question over whether Lp-PLA2 actually has a functional role in the development of atherosclerosis, or is simply marking the disease state. This might explain why Lp-PLA2 activity/mass measures have been found to be consistently associated with CHD risk (Caslake et al., 2000; Blake et al., 2001; Blankenberg et al., 2003; Ballantyne et al., 2004; Winkler et al., 2005; Oei et al., 2005; Koenig et al., 2004), yet an association of genotype with CHD risk has not been apparent in the EPIC-Norfolk and NPHS II studies. As discussed in the introduction of this chapter, reproducibility of genetic association studies is often difficult (Hirschhorn and Altshuler, 2002; Ioannidis et al., 2001), and in more modestly sized studies such as HIFMECH there could have been an over-estimation of the size of the effect of the A379V variant (Colhoun et al., 2003; Farrall and Morris, 2005), or even the possibility of a type I error (Cardon and Bell, 2001)(although the replication of this association in the larger AtheroGene study would suggest otherwise).

The final possibility is that epidemiological studies of CHD risk such as EPIC-Norfolk and NPHS II are unable to distinguish the pro- and anti- atherogenic properties of the Lp-PLA2 enzyme, since they only investigate global measures of Lp-PLA2 activity and CHD. The A379V variant could be having a more qualitative effect on enzyme function by altering substrate specificity of the Lp-PLA2 enzyme, shifting the balance between a pro- and anti- atherogenic function. It is clear that further *in vitro* and *in vivo* work is needed to characterise the functional effect of the A379V variant before undertaking large-scale epidemiological analyses with confidence. If the variant or haplotype under investigation does not show a consistent effect on the function of an enzyme, then reproducing previous associations will always be challenging (Hirschhorn and Altshuler, 2002).

4.3.5 Summary of EPIC-Norfolk results

i) In agreement with the NPHS II data, higher Lp-PLA2 activity was associated with higher levels of lipid markers (lower levels of HDL) in both male and female participants of the EPIC-Norfolk study.

ii) Quartiles of Lp-PLA2 activity showed a positive association with CHD risk which was lost after subsequent adjustment for cholesterol and HDL.

iii) In agreement with the NPHS II study, individuals homozygous for the 379V allele showed a significantly higher Lp-PLA2 activity compared to AV and AA men and women.

iv) There was a borderline significant association of 379VV individuals with lower HDL levels compared to AV and AA individuals

v) There was no significant association of A379V individuals with CHD risk. In a recessive model, those homozygous for the 379V allele showed a borderline significant higher CHD risk, although this lost significance after suitable adjustment.

4.4 Association of the *PLA2G7* G-1230A variant with risk in the HIFMECH case-control study

4.4.1 Introduction

To the best knowledge of the author, associations of the novel *PLA2G7* G-1230A gene variant with CHD risk have not been tested in any large epidemiological studies, therefore meriting the investigation of this SNP with CHD risk in a case-control or prospective study. The NPHS II study represents a well-powered prospective cohort for examining risk and measured traits over time. Unfortunately the relatively small number of cases in the NPHS II study, combined with the rare allele frequency (0.19) of the A379V genotype made it difficult to conclude whether the *PLA2G7* A379V variant was associated with risk: for an 80% power of detection and a 0.05 two-sided level of significance, the NPHS II study had the power to detect a relative risk of <0.57 or >1.62 for the A379V variant. It was therefore important to take this into consideration when genotyping other variants with a similar rare allele frequency.

In order to minimise the possibility of a false-negative association of the G-1230A variant with CHD risk, I decided to test the association of this variant with CHD risk using the Hypercoagulability and Impared Fibrinolytic function MECHanisms study (HIFMECH). The HIFMECH study was originally designed to identify differences in genetic and environmental risk markers for myocardial infarction (MI) between subjects living in the North and South of Europe (Mannila et al., 2004; Juhan-Vague et al., 2002). An advantage of the HIFMECH cohort is that it represents a powerful case-control study consisting of 533 post-MI patients under the age of 60 (cases) and 575 controls matched by age and geographical region. Assuming a rare allele frequency of 0.20 (similar to the G-1230A allele frequency observed in other studies genotyped for this thesis), 80% power of detection, and a 0.05 two-sided level of significance, the HIFMECH study is able to detect a relative risk of <0.64 and >1.49; therefore making this a more powerful study than the NPHS II study in detecting an association of genotype with CHD risk. Genotyping the G-1230A variant in the HIFMECH study would help determine the relationship of this SNP with CHD risk, and further characterise the functional role of the Lp-PLA2 enzyme in the progression of atherosclerosis.

4.4.2 Materials and methods

4.4.2.1 The Hypercoagulability and Impared Fibrinolytic function MECHanisms study (HIFMECH)

Age matched healthy, Caucasian individuals and post-infarction patients who survived a first MI event below 60 years of age were recruited from four centres in Europe. Two of these centres, Stockholm, Sweden and London, England were taken to represent the North European region while the remaining two centres, Marseille, France and San Giovanni Rotondo, Italy, represented a Southern European region. Patients with familial hypercholesterolemia and insulin-dependent diabetes mellitus were excluded from the study. Consecutive patients along with randomly selected healthy individuals from the same area were invited to participate. A total of 598 MI survivors and 653 healthy control subjects were included in the initial study. In the current study, only those with partial or complete environmental data were considered and in total, 533 cases and 575 controls were recruited. Matching of controls to cases was done on the basis of centre and age. While the study design set out to recruit one to one matching, a few more controls to patients were recruited in some centres. Survivors were examined 3-6 months after MI. Examination of both patient and control subjects, was performed in the early morning following an overnight fast. A structured interview concerning socio-economic and lifestyle information was carried out together with a clinical examination involving weight, height, waist and hip circumference measurements, systolic and diastolic blood pressure measurements. Blood samples were taken after 20-minutes rest in the recumbent position, for plasma and DNA analysis. All subjects gave informed consent to their participation, and the local Ethics committees of the four centres approved the study.

4.4.2.2 G-1230A genotyping

Oligonucleotides for the G-1230A polymorphism were designed using Primer 3 software, and are shown in Table 2.1, along with the relevant restriction enzyme conditions required. Section 2.2 of the material and methods chapter describes the complete PCR and digestion process used for genotyping the HIFMECH study.

4.4.2.3 Statistical analysis

Data was entered onto an EXCEL spreadsheet (Microsoft) and tested for deviation from Hardy-Weinberg equilibrium by using a χ^2 test. Statistical analysis was performed by Jackie Cooper, our statistician, using STATA (Intercooled STATA Version 8.0, STATA Corp.). Continuous variables are expressed as means ± 1 standard deviation

(±1SD) for normally distributed variables, or geometric means and approximate SD for variables where normal distribution was reached after a log or square root transformation. Normality was considered graphically with QQ-norm plots and formally via a Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was performed to compare differences in continuous variables by genotype/case-control status, and transformed variables were used as appropriate. Differences in the frequency of categorical variables were examined using Pearson χ^2 tests. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) as an estimate of the relative risk of incident CHD were calculated using conditional logistic regression analysis taking into account lifestyle factors and inflammatory markers.

4.4.3 Results

4.4.3.1 Baseline Characteristics by CHD status

Table 4.4.1 lists the baseline characteristics of the HIFMECH study by CHD status. CHD cases had higher BMI readings ($p<0.0005$), were more likely to be smokers ($p<0.0005$), and had higher plasma TG levels ($p<0.0005$). Fibrinogen, IL-6, CRP and the number of individuals with diabetes were also higher in the CHD-case group (all $p<0.0005$). Cholesterol levels were lower in cases than controls ($p=0.04$), but this was not surprising since 27% of all cases were on lipid lowering treatments. Diastolic blood pressure was also lower in the cases than controls ($p<0.0005$), potentially due to the fact that 84% of cases were on anti-hypertensive drugs.

Table 4.4.1 Baseline characteristics of the HIFMECH study by case-control status

Trait	Controls	Cases	P value
Age (years)	51.5 (5.4)	51.9 (5.4)	0.18
BMI kg/m^2 ‡	26.1 (3.2)	27.1 (3.3)	<0.0005
Systolic BP mmHg ‡	127.9 (14.4)	127.9 (17.0)	0.96
Diastolic BP mmHg ‡	84.1 (8.5)	81.8 (10.3)	<0.0005
Current and ex-smokers %	61.2	82.2	<0.0005
Never Smokers %	37.8	17.8	
T2DM %	0	11.3	<0.0005
Cholesterol mmol/L	5.52 (0.97)	5.39 (1.18)	0.04
Triglycerides mmol/L ‡	1.44 (0.61)	1.87 (0.77)	<0.0005
Fibrinogen mg/dL+	340.2 (69.1)	371.2 (97.1)	<0.0005
IL-6 pg/ml ‡	1.24 (0.78)	1.97 (1.34)	<0.0005
CRP mg/L +	1.45 (1.41)	2.37 (2.51)	<0.0005

‡ Natural log geometric mean. SD is approximate + square of square root transformed mean. SD is approximate.

4.4.3.2 Baseline characteristics by G-1230A genotype in control subjects

The G-1230A genotype did not significantly differ from Hardy Weinberg in any of the four centres and in combined analysis ($p>0.15$). None of the traits tested in HIFMECH differed by G-1230A genotype in either the case or control group (Table 4.4.2). In addition, no heterogeneity of effect existed between the case and control group ($p>0.11$ for all traits measured).

Table 4.4.2: Baseline characteristics by G-1230A genotype in the HIFMECH study split by CHD status. All values are adjusted for centre differences

Trait	G1230A	Controls N=575	Cases N=533
Age, years	**GG**	52.6 (5.3)	52.3 (5.4)
	GA	52.7 (5.5)	52.5 (5.1)
	AA	53.1 (5.5)	53.9 (4.9)
	P value	**0.90**	**0.16**
BMI, kg/m^2 *	**GG**	25.7 (3.1)	27.3 (3.4)
	GA	25.9 (3.2)	27.2 (3.3)
	AA	26.5 (3.3)	26.5 (2.9)
	P value	**0.38**	**0.48**
Smoking (% current/ex)	**GG**	238 (61.5)	278 (81.5)
	GA	97 (64.7)	130 (82.8)
	AA	14 (51.9)	27 (84.4)
	P value	**0.43**	**0.89**
SBP, mmHg *	**GG**	130.1 (14.7)	129.8 (17.6)
	GA	130.2 (13.7)	128.5 (16.1)
	AA	132.5 (16.6)	131.7 (17.5)
	P value	**0.72**	**0.58**
DBP, mmHg	**GG**	84.0 (8.5)	81.9 (10.5)
	GA	83.8 (8.7)	82.1 (9.7)
	AA	84.0 (8.0)	85.5 (9.6)
	P value	**0.95**	**0.16**
Cholesterol, mmol/L	**GG**	5.75 (0.95)	5.64 (1.08)
	GA	5.57 (0.94)	5.65 (1.33)
	AA	5.92 (1.14)	5.88 (1.03)
	P value	**0.08**	**0.55**
Triglycerides mmol/L *	**GG**	1.49 (0.60)	1.97 (0.80)
	GA	1.60 (0.73)	2.10 (0.82)
	AA	1.52 (0.65)	2.11 (0.78)
	P value	**0.25**	**0.61**
Fibrinogen, mg/dL +	**GG**	341.9 (67.9)	380.0 (88.8)
	GA	346.4 (74.3)	376.6 (99.6)
	AA	325.5 (61.4)	348.6 (95.9)
	P value	**0.36**	**0.20**
CRP, mg/L +	**GG**	0.92 (1.01)	2.07 (2.36)
	GA	0.90 (1.06)	2.03 (2.34)
	AA	0.96 (0.81)	2.38 (2.69)
	P value	**0.95**	**0.79**

*Log transformed. SD is approximate. + Square root transformed. SD is approximate

4.4.3.3 Genotype and allele frequencies by centre and case-control status

Since the HIFMECH study was recruited from 4 different centres across Europe, it was important to investigate whether allele frequencies were significantly different by centre in both cases and controls. When considered separately, allele and genotype

frequencies from the different centres in the controls did not significantly differ from each other (p=0.08 and p=0.17, respectively). This was also found in the case group, with no differences in allele or genotype frequency (p=0.30 and p=0.53, respectively)(Table 4.4.3).

4.4.3.4 Odds ratio for the G-1230A variant

Table 4.4.4 shows the odds ratios (OR) of the G-1230A variant by centre. Although there was no significant association of this variant with CHD risk in individual centres, there did appear to be a trend for those individuals homozygous for the -1230A allele to have a higher risk of CHD. The odds ratios did not significantly differ by centre (p=0.81), enabling all the centres to be examined as one group. In combined analysis there was no significant association of the G-1230A SNP with CHD risk, although the same trends seen in individual centres appeared to exist. Adjustment for lifestyle factors, BMI and the inflammatory marker CRP did not appear to alter these results significantly.

The -1230A allele was found to be relatively rare in the HIFMECH study, therefore by combining the -1230A homozygous and heterozygous groups, it was hoped that the power of the study could be increased and a potentially weak association with CHD risk detected. In agreement with the previous analysis, carriers of the -1230A allele showed a non-significant trend towards higher CHD risk within individual centres. When the centres were combined, there appeared to be a trend for higher risk of CHD in those carriers of the -1230A allele (OR GG=1, GA/AA= 1.26 (95%CI 0.97-1.65), p=0.09), however, adjustment for lifestyle factors, BMI and CRP weakened this trend (p>0.60) (Table 4.4.5 and Fig 4.4.1).

Table 4.4.3 Genotype and allele frequencies by Centre and Case-control status

	CONTROLS				CASES			
Centre	**GG**	**GA**	**AA**	**Allele freq (95% CI)**	**GG**	**GA**	**AA**	**Allele freq (95% CI)**
Stockholm	115 (66.1)	52 (29.9)	7 (4.0)	0.19 (0.15-0.23)	114 (63.7)	52 (29.1)	13 (7.3)	0.22 (0.18-0.26)
London	48 (68.6)	19 (27.1)	3 (4.3)	0.18 (0.12-0.25)	35 (64.8)	17 (31.5)	2 (3.7)	0.19 (0.12-0.28)
Marseilles	93 (76.2)	27 (22.1)	2 (1.6)	0.13 (0.09-0.18)	69 (69.7)	28 (28.3)	2 (2.0)	0.16 (0.11-0.22)
Italy (SGR)	131 (66.2)	52 (26.3)	15 (7.6)	0.21 (0.17-0.25)	123 (62.1)	60 (30.3)	15 (7.6)	0.23 (0.19-0.27)
Total	387 (68.6)	150 (26.6)	27 (4.8)	0.18 (0.16-0.20)	341 (64.3)	157 (29.6)	32 (6.0)	0.21 (0.18-0.23)

Controls: P value for difference in genotype frequency by centre p=0.17
Controls: P value for difference in allele frequency by centre p=0.08.
Cases: P value for difference in genotype frequency by centre p=0.53
Cases: P value for difference in allele frequency by centre p=0.30

Table 4.4.4: Odds Ratios for G-1230A. Controls are matched to cases within centre so analysis takes account of centre differences

Centre		Unadjusted	Adjusted[1]	Adjusted[2]
Stockholm	GG	1.00	1.00	1.00
	GA	1.02 (0.65-1.62)	0.88 (0.51-1.52)	0.87 (0.48-1.58)
	AA	1.73 (0.67-4.46)	1.60 (0.50-5.07)	1.13 (0.34-3.77)
	P value	P=0.51	P=0.58	P=0.86
London	GG	1.00	1.00	1.00
	GA	1.93 (0.69-5.40)	1.57 (0.38-6.46)	2.65 (0.50-13.97)
	AA	1.18 (0.16-8.65)	0.92 (0.02-37.78)	0.83 (0.01-86.05)
	P value	P=0.44	P=0.82	P=0.50
Marseilles	GG	1.00	1.00	1.00
	GA	1.64 (0.81-3.31)	1.21 (0.39-3.77)	0.90 (0.32-2.58)
	AA	1.94 (0.26-14.48)	1.36 (0.06-30.65)	1.72 (0.07-39.40)
	P value	P=0.33	P=0.93	P=0.93
Italy (SGR)	GG	1.00	1.00	1.00
	GA	1.24 (0.79-1.96)	1.04 (0.54-1.99)	0.98 (0.50-1.92)
	AA	1.07 (0.52-2.23)	1.37 (0.50-3.79)	1.30 (0.43-4.000
	P value	P=0.65	P=0.83	P=0.89
Total	GG	1.00	1.00	1.00
	GA	1.25 (0.94-1.66)	1.06 (0.75-1.48)	1.11 (0.78-1.58)
	AA	1.33 (0.78-2.27)	1.29 (0.67-2.47)	1.35 (0.70-2.62)
	P value	P=0.22	P=0.73	P=0.61

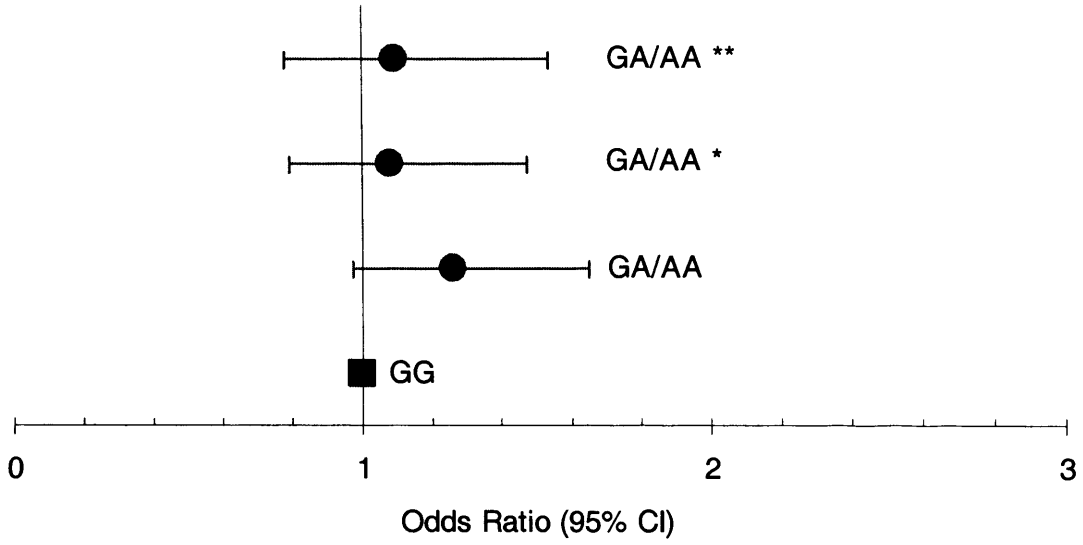
[1] adjusted for BMI, smoking, alcohol, exercise.
[2] as 1 + CRP.
Odds ratios do not differ by centre p=0.81

Table 4.4.5: Odds Ratio for combined GA /AA -1230 genotypes. Controls are matched to cases within centre so analysis takes account of centre differences

Centre		Unadjusted	Adjusted[1]	Adjusted[2]
Stockholm	GG	1.00	1.00	1.00
	GA/AA	1.11 (0.71-1.71)	0.95 (0.56-1.61)	0.94 (0.52-1.67)
	P value	P=0.66	P=0.85	P=0.82
London	GG	1.00	1.00	1.00
	GA/AA	1.77 (0.68-4.61)	2.44 (0.55-10.75)	3.02 (0.50-18.12)
	P value	P=0.24	P=0.24	P=0.23
Marseilles	GG	1.00	1.00	1.00
	GA/AA	1.66 (0.84-3.29)	1.33 (0.48-3.64)	0.95 (0.35-2.59)
	P value	P=0.15	P=0.58	P=0.92
Italy (SGR)	GG	1.00	1.00	1.00
	GA/AA	1.20 (0.79-1.83)	1.06 (0.60-1.87)	1.04 (0.57-1.90)
	P value	P=0.39	P=0.84	P=0.90
Total	GG	1.00	1.00	1.00
	GA/AA	1.26 (0.97-1.65)	1.08 (0.79-1.47)	1.09 (0.78-1.53)
	P value	P=0.09	P=0.64	P=0.60

[1] adjusted for BMI, smoking, alcohol, exercise.
[2] as for 1 + CRP.

Fig 4.4.1: Odds Ratio graph for the combined GA/AA -1230 association with CHD risk. Narrow bars represent 95%CI. *is adjustment for lifestyle factors and BMI. **is adjustment for lifestyle factors, BMI and CRP.



4.4.4 Discussion

Analysis of the A379V variant in both the NPHS II and EPIC-Norfolk studies produced inconsistent associations with CHD risk when compared to previously published data (Abuzeid et al., 2003; Ninio et al., 2004). Chapter 3 of this thesis highlighted the successful detection of a novel G-1230A SNP in the promoter region of the *PLA2G7* gene which had not been investigated in the AtheroGene based haplotype analysis (Ninio et al., 2004), and whose functional effect had not been characterised. In an effort to further characterise the G-1230A SNP, it was necessary to test the association of this novel variant with CHD risk in the HIFMECH study.

An advantage of the HIFMECH study in comparison to that of the NPHS II study was the considerable power that it gave to detect any potential association with CHD risk. Unfortunately, the HIFMECH study was originally designed to investigate differences in Northern and Southern Europe and can be thought of as the merging of 4 different population groups (Mannila et al., 2004; Juhan-Vague et al., 2002). This could lead to problems (already discussed in the introduction of this chapter) concerning population stratification (Suh and Vijg, 2005), and also the possibility that variability in environmental conditions between Northern and Southern European samples could affect the association of genotype with CHD risk (Colhoun et al., 2003). Despite these issues being particularly relevant to the HIFMECH study design, the allele frequency of the G-1230A SNP was not significantly different by centre in both control ($p=0.08$) and case ($p=0.30$) groups, and the association of odds ratio with genotype did not significantly differ ($p=0.81$) between the four centres. Therefore, the lack of observed heterogeneity enabled all four centres to be combined, increasing the power of the study.

Considering the association of G-1230A genotype with intermediate phenotypes, no significant associations were found in either the case or control group. However, the main aim of using the HIFMECH study was to investigate the association of this genotype with CHD risk. Association of G-1230A genotype with traits which could be modified by the Lp-PLA2 enzyme would be addressed in more detail in the UDAC and BH2 studies (section 4.4 and 4.5). Individual analysis of the G-1230A SNP showed that there was no association of genotype with CHD risk in any of the centres, or in combined analysis. Despite this, those homozygous for the -1230A allele displayed a non-significant trend for higher CHD risk in all four centres and in combined analysis ($p=0.22$). Additional adjustment for BMI, smoking, alcohol, exercise and CRP did not

appear to alter this trend. The small number of -1230A homozygous individuals (n=59) attenuated some of the power of the HIFMECH study. By combining those GA and AA homozygous individuals in a recessive model, it was hoped that there would be added power in the study. Indeed, those carriers of the -1230A allele showed a borderline trend towards higher CHD risk compared to G-1230 homozygous individuals (p=0.09), although this trend was lost after adjustment for lifestyle factors and CRP.

Chapter three of this thesis demonstrated that the G-1230A SNP is in strong negative LD with the A379V variant (D' =-0.90, p<0.0005). As a consequence, the non-significant trend of G-1230A genotype with CHD risk seen in HIFMECH could be a weakened reflection of the previously reported significant association of A379V genotype with CHD risk (Abuzeid et al., 2003). There is however the possibility that one or both of these SNPs could be in LD with an as yet undefined functional variant, or be part of a functional haplotype. The failure to find an association of the G-1230A SNP with CHD risk does not fully rule out a functional role, since analysis of single rare variants exhibiting modest effects are invariably not sufficiently common to permit an accurate assessment of CHD risk (Hirschhorn and Altshuler, 2002). While the G-1230A SNP doesn't appear to be associated with CHD risk, it could be having a modest effect on the rate of transcription of the *PLA2G7* gene, which in combination with other rare variants could exert a significant effect on Lp-PLA2 function. In spite of previous research which has demonstrated the A379V variant to be independently associated with CHD risk (Ninio et al., 2004), the unconvincing results observed from the EPIC-Norfolk and NPHS II studies suggest that further characterisation of the genetic variation present in the *PLA2G7* gene is warranted. Therefore, the G-1230A SNP would be genotyped in two additional studies of differing design in order to investigate association of this variant with traits that are potentially modified by the Lp-PLA2 enzyme. In parallel to these investigations, promoter activity assays would be used to determine the independent functional effect of this variant, and are described in more detail in chapter 5.

4.5 Association of *PLA2G7* variants and Lp-PLA2 activity with markers of atherosclerosis in a population of diabetic individuals

4.5.1 Introduction

The UCL Diabetes and Cardiovascular study (UDAC) is a cross-sectional design of mixed type I and type II diabetic individuals, originally developed to investigate the relationship between markers of oxidative stress (and other associated risk factors) and diabetes. Diabetes is regarded as a serious risk factor in its own right for CHD (Garcia et al., 1974; Panzram, 1987), with the MRFIT trial showing that those individuals with T2DM who are non-smokers, normotensive with normal serum cholesterol, having the same risk from CHD as a non-diabetic patient who has two to three risk factors present (Stamler et al., 1993). Several studies have also found that those individuals with features of the metabolic syndrome (MS) are associated with an increased risk of both diabetes (Grundy et al., 2004) and CHD (Isomaa et al., 2001; Lakka et al., 2002). Most current definitions of the MS involve several conditions such as: Insulin resistance, obesity, dyslipidaemia, hypertension, and lowered glucose tolerance (Eckel et al., 2005). However, Insulin resistance is widely accepted as being the unifying hypothesis with regards to the pathophysiology of the MS (Eckel et al., 2005). As such, diabetes and the metabolic syndrome are interesting phenotypes to investigate since both these conditions share common genetic and environmental stresses with CHD, in particular, those mechanisms involving oxidative stress (Ceriello and Motz, 2004).

As discussed in chapter 1, the progression of atherosclerosis is intimately associated with the oxidative modification of plasma lipoproteins by free radicals and other enzymatic processes. The association of Lp-PLA2 with LDL, via an interaction with apolipoprotein (apo) B (Stafforini et al., 1999), makes this enzyme ideally placed to participate in these key oxidative steps. Lp-PLA2 has a marked substrate preference for choline-containing phospholipids (phosphatidylcholine is the major phospholipid component of LDL particles (Hevonoja et al., 2000)) including short fatty acid chain oxidised phospholipids, both of which interact with G-protein-coupled receptors, leading to inflammatory cell activation (Marathe et al., 2001). In this way, Lp-PLA2 mediated breakdown of oxidatively damaged phospholipids may protect LDL particles from involvement in the progression of atherosclerosis. However, Lp-PLA2 is also associated with the generation of significant amounts of lyso-phosphatidylcholine (lyso-PC) and free oxidised fatty acids from the degradation of oxidised phosphatidylcholine

(oxPC) (Karabina et al., 1997; Macphee et al., 1999), with both of these products exerting pro-atherogenic effects.

The prospective NPHS II, case-control designed EPIC-Norfolk and HIFMECH studies are powerful studies that were used in this thesis to investigate the relationship between Lp-PLA2 activity, genotype and CHD risk. However, these cohorts were not designed with fully comprehensive measures of intermediate phenotypes that may be affected by Lp-PLA2. By contrast, the UDAC study has measures of oxidative stress and oxidised LDL (oxLDL), both of which could be mediated by the Lp-PLA2 enzyme. The UDAC study has limited power to investigate whether Lp-PLA2 activity or genotype are associated with CHD risk, although there are other measures related to CHD risk such as the metabolic syndrome, and a unique diabetic risk algorithm similar to that of FRAMINGHAM (Stevens et al., 2001).

Any observed associations of Lp-PLA2 activity and genotype with intermediate phenotypes of oxidative stress and LDL measures may become more apparent in those individuals at greater risk of CHD i.e. those individuals with the MS and diabetes. Therefore, to investigate further the association of Lp-PLA2 with traits such as total anti-oxidant status and LDL oxidation, the UDAC study was genotyped for both the *PLA2G7* A379V and G-1230A variants as well as being assayed for Lp-PLA2 activity.

4.5.2 Materials and methods

4.5.2.1 Study Design

All subjects were recruited between December 2001 and January 2003 from the diabetes clinic at University College London Hospitals NHS Trust (UCLH). Clinical information was gathered from the computerised clinic database which contains demographic and clinical information on patients attending the diabetes clinic. All patients had diabetes according to WHO criteria (Alberti and Zimmet, 1998). From the database, patients were categorised by the presence/absence of clinically manifest CHD. Table 4.5.1 summarises the characteristics of the UDACS sample. Analysis in this thesis will focus on Caucasian subjects only, as these individuals made up the majority of the sample (approximately 80%). Furthermore, the frequency of gene variants may differ between different ethnic groups, and subjects from certain racial origins (e.g. South India) with diabetes are at an increased risk of CHD. (Of note, within the sample, the next most common ethnic group were those from the Indian continent, and comprised 11% of the sample). Demographic data including gender, smoking history, family history, medical treatment (diabetes and other), age, date of diagnosis, and type of diabetes was taken. All subjects were free from acute illnesses at the time of recruitment. Ethical approval was obtained from UCL/UCLH Ethics Committee and the project was registered with the Department of Research and Development at UCLH.

Table 4.5.1: The University College London Diabetes And Cardiovascular Disease Study (UDACS)

Cohort of 1011 subjects with diabetes	
Age (years)	61.8 (13.2)
Duration of diabetes (years)	11 (5-19)
Type of diabetes	
I	17.8% (n=180)
II	79.1% (n=800)
Pancreatic	3.1% (n=31)
CHD	20.1% (n=198)
Caucasian	77% (n=780)
Males	62% (n=631)

4.5.2.2 Definitions and Clinical measures

The presence of CHD was recorded if any patient had positive coronary angiography/ angioplasty, coronary artery bypass, cardiac thallium scan, exercise tolerance test, myocardial infarction or symptomatic/treated angina. Any individual who was asymptomatic or had negative investigations was categorised as 'no CHD'. Individuals suffering from the metabolic syndrome (MS) in UDACS were defined under the World Health Organisation classification (Alberti and Zimmet, 1998) as exhibiting the following: a systolic BP greater than 130mmHg and a diastolic BP over 80mmHg; those on hypertensive therapy; triglyceride levels higher than 1.7mmol/L; an HDL level lower than 1.0mmol/L (1.29mmol/L in females); a BMI greater than 30kg/m^2; and all had clinically defined Diabetes. The UK Prospective Diabetes Study (UKPDS) risk algorithm was also applied to each participant at the beginning of the study and is comparable to that of the Framingham risk assessment, yet is specifically aimed at individuals with diabetes. The algorithm incorporates glycaemia, blood pressure, and lipid levels as risk factors in addition to age, sex, ethnic group, and smoking status (Stevens et al., 2001). The score was ranked out of 100, with the maximum rating being given to those people with a CHD event. Clinic measurements of blood pressure (supine and lying), weight and height were measured on all subjects. Routine clinic biochemistry was also recorded including HbA_1c, random glucose, total cholesterol, LDL, HDL, random triglycerides, thyroid function, albumin:creatinine ratio, and proteinuria by dipstick. Routine plasma traits were measured including plasma oxidised LDL by ELISA (the antibodies used are targeted to the ApoB protein) (Scheffer et al., 2003). The inter-assay CV was 14%, and the intra-assay CV was 10% in this study. Oxidised LDL measures were then divided by total LDL in order to generate a specific measure of LDL oxidation (Scheffer et al., 2003). Plasma Total Anti-Oxidant Status (TAOS) is a measure of oxidative stress and was measured using a photometric micro-assay as previously described (Sampson et al., 2002). Lp-PLA2 was kindly measured by Ewa Ninio (INSERM-Paris) by methods already described in this chapter (mean intra-assay CV was 3.5% and inter-assay CV of 34.7%).

4.5.2.3 *PLA2G7* A379V and G-1230A genotyping

Genotyping was carried out by PCR and restriction enzyme digestion as described previously in this chapter and chapter 2 (section 2.2). Table 2.1 lists the conditions used for the assays.

4.5.2.4 Statistical Analysis

All statistical analysis was conducted by myself using SPSS v12.1. Departure from Hardy-Weinberg equilibrium was assessed using chi-squared tests as already described. LD was assessed by Δ and D'. All analyses were performed on normally distributed data after appropriate transformation (log or square root). A univariate step-wise regression model was subsequently applied to assess contributors to variance of Lp-PLA2 activity. Differences in continuous variables were examined using analysis of variance (ANOVA) and linear trend ($p<0.05$ was taken as statistically significant). Where adjustments were required, analysis of covariance was applied. For analysis of oxLDL/LDL and UKPDS risk score by *PLA2G7* genotype, women were included as there was no heterogeneity among sexes with regards to genotype effects on either of these variables.

4.5.3 Results

4.5.3.1 Baseline characteristics of the UDAC study

Table 4.5.2 shows the baseline characteristics of the Caucasian subjects (men and women) in the UDAC study in those with or without CHD. As expected, those men who were positive for CHD were older, more obese, had lower HDL, and higher triglycerides and CRP levels. They also showed a higher usage of statins and ACE inhibitors, potentially explaining their lower diastolic blood pressure, and LDL-C levels. Those women positive for CHD were older, and had a higher usage of statins, possibly explaining their lower LDL-C levels. Lp-PLA2 activity was significantly *lower* in those diabetic men with CHD (31.0± 10.5 nmol/min/ml) than those without (34.2± 11.3 nmol/min/ml, p= 0.005). However, this was no longer significant after adjustment for age, BMI, total cholesterol, and drug usage (ACE inhibitors and Statins) (p=0.38). There was no evidence that either type of Diabetes or CHD status interacted with oxLDL/LDL, UKPDS risk score, Metabolic syndrome (MS) or statin use, in their effect on Lp-PLA2 activity. Type of Diabetes, CHD status, and statin use were adjusted for in the following analysis.

Table 4.5.2: Baseline characteristics of the Caucasian individuals in the UDAC study by CHD status.

	Males			Females		
	No CHD (n=367)	CHD (n=107)	p value	No CHD (n=258)	CHD (n=51)	p value
Age	59.3 (13.8)	67.5(11.2)	<0.0005	63.3 (14.1)	70.5 (8.4)	0.001
Type I Diabetic %	29.2%	4.8%	<0.0005	23.6%	5.9%	0.002
BMI Kg/m^2 ‡	28.1 (5.0)	29.3 (5.7)	0.03	28.4 (6.2)	29.6 (4.6)	0.19
Systolic BP mmHg ‡	137.4 (18.6)	136.5 (23.8)	0.68	140.4 (20.5)	142.2 (17.5)	0.55
Diastol. BP mmHg ‡	81.3 (11.3)	76.6 (12.5)	<0.0005	77.6 (10.4)	76.6 (9.8)	0.55
LDL mmol/L+	2.8 (0.8)	2.2 (0.6)	<0.0005	2.9 (0.7)	2.5 (0.7)	0.01
oxLDL mU/L+	46.3 (17.9)	45.3 (20.0)	0.67	48.7 (17.9)	47.6 (17.4)	0.75
oxLDL/LDL U/mmol*	16.9 (7.1)	19.3 (11.5)	0.04	16.7 (7.8)	18.4 (6.5)	0.25
HDL mmol/L ‡	1.8 (0.6)	1.4 (0.4)	<0.0005	1.5 (0.5)	1.4 (0.4)	0.16
TG mmol/L ‡	1.6 (1.0)	1.9 (1.1)	0.03	1.7 (1.0)	1.9 (0.9)	0.12
CRP mg/L ‡	1.4 (1.2)	1.8 (1.5)	0.005	1.8 (1.5)	1.8 (1.7)	0.92
Statin use %	15	63	<0.0005	23	58	<0.0005
ACE I %	43	63	<0.0005	41	41	0.95
Lp-PLA2 activity nmol/min/ml *	34.2 (11.3)	31.0 (10.5)	0.005	na	na	na
A 379V Rare Allele frequency	0.20 (0.17-0.23)	0.21 (0.16-0.27)	0.91	0.21 (0.17-0.25)	0.23 (0.15-0.31)	0.62
G-1230A Rare allele frequency	0.20 (017-0.23)	0.19 (0.14-0.24	0.84	0.20 (0.16-0.23)	0.22 (0.14-0.31)	0.66

*antilog of log10 transformed mean. SD is approximate. ‡ Natural log geometric mean. SD is approximate + square of square root transformed mean. SD is approximate. Allele frequencies are given with 95%CI in brackets

4.5.3.2 Metabolic syndrome and UKPDS risk score in relation to Lp-PLA2 activity

Considering the relationship between those men with features of the metabolic syndrome (MS) and Lp-PLA2 activity, men with the MS (n=205) had a higher Lp-PLA2 activity (35.6± 11.9nmol/min/ml) compared to those without the MS (n=206; 33.0± 10.8nmol/min/ml) after adjustment for type of Diabetes, CHD status and statin use (Table 4.5.3, p=0.02). When considering men with CHD and those without separately (Fig. 4.5.1), the effect remained significant in those diagnosed as negative for CHD (p=0.008), however, there was no longer a significant difference in activity in those positive for CHD(p=0.98); both results were adjusted for type of diabetes and statin use. In addition, when the UKPDS risk algorithm score (Stevens et al., 2001) was divided into quartiles of risk and compared against Lp-PLA2 activity, there was a positive relationship between UKPDS CHD risk score and higher Lp-PLA2 activity (p=0.006 and p=0.004 for linear trend)(Table 4.5.3) after adjustment for CHD status, diabetes type, and statin use. This effect was borderline significant in those without CHD (p=0.08), while remaining significant in those positive for CHD (p=0.01)(Fig. 4.5.2).

4.5.3.3 Oxidised LDL in relation to Lp-PLA2 activity

Oxidised LDL was not significantly different in those with or without CHD in men or women. However, the oxLDL level relative to LDL concentration (oxLDL/LDL) was significantly higher in those men with the presence of CHD (Table 4.5.2). Those men in the highest quartile of oxLDL/LDL level had the lowest Lp-PLA2 activity (31.3± 10.5 nmol/min/ml) when compared to the middle two (32.3 ±9.8nmol/min/ml, and 35.9±10.9 nmol/min/ml respectively) and lowest quartile (35.6 ±12.5 nmol/min/ml)(p=0.03, p=0.004 linear trend) after adjustment for type of diabetes, CHD status and statin use(Table 4.5.3). The effect was even more significant in those who did not have the presence of CHD(p=0.008), and was no longer seen in those with the presence of CHD(p=0.37)(Fig. 4.5.3).

4.5.3.4 Association of Lp-PLA2 activity with TAOS measures

In the UDAC study, Lp-PLA2 activity measures did not correlate with %TAOS (r=-0.003, p=0.96). Consequently, when quartiles of %TAOS were considered, there was no significant association (p=0.19 and p=0.53 linear trend) with Lp-PLA2 activity measures (Table 4.5.3).

4.5.3.5 Association of Lp-PLA2 activity with Statin use

Interestingly, those individuals receiving statin therapy had significantly lower Lp-PLA2 activity (31.5± 12.6 nmol/min/ml) compared to those not receiving treatment (33.9± 11.8 nmol/min/ml), even after adjustment for type of diabetes and CHD status (Table 4.5.3, p=0.04). However, additional adjustment for LDL led to the loss of this association (p=0.56).

Table 4.5.3: Geometric mean for LpPLA2 in Caucasian men adjusted for diabetes type, CHD status and statin use (except for the statin comparison where the result was adjusted for by CHD status and diabetes use only).

	N	Adjusted mean(±1SD)	Adjusted p value
Metabolic syndrome			
No	206	33.0 (10.8)	P=0.02
Yes	205	35.6 (11.9)	
Oxidised LDL/LDL U/mmol			
Quartile 1 (0-13.6)	75	35.6 (12.5)	P=0.03
Quartile 2 (13.6-17.7)	66	35.9 (10.9)	(p=0.004 linear trend)
Quartile 3 (17.7-22.1)	65	32.3 (9.8)	
Quartile 4 (>22.1)	74	31.3 (10.5)	
UKPDS			
Quartile 1 (0-13.7)	69	33.4 (11.5)	P=0.006
Quartile 2 (13.7-22.4)	73	32.2 (11.2)	(p=0.004 linear trend)
Quartile 3 (22.4-36.7)	104	32.9 (10.5)	
Quartile 4 (>36.7)	127	37.4 (12.5)	
% TAOS			
Quartile 1 (0-35.8)	102	34.3 (12.9)	P=0.19
Quartile 2 (35.8-44.5)	110	32.4 (11.3)	(p=0.53 linear trend)
Quartile 3 (44.5-51.7)	103	32.2 (12.2)	
Quartile 4 (>51.7)	112	35.0 (12.6)	
Statin Therapy			
No	319	33.9 (11.8)	P=0.04
Yes	110	31.5 (12.6)	

4.5.3.5 A379V and G-1230A individual genotype associations

Table 4.5.2 lists allele frequencies for both the G-1230A and A379V SNP in Caucasian individuals by CHD status in males and females. When both CHD and CHD free groups were considered together, neither SNP departed from Hardy Weinberg Equilibrium, and both SNPs were found to be in negative LD when measured by Δ (-0.24, $p<0.005$) and D' (-0.92, $p<0.005$). Both values are consistent with other studies genotyped in this thesis. Although Lp-PLA2 activity was only measured in men, genotype data was available for both men and women. Table 4.5.4 and 4.5.5 show the baseline characteristics of the UDAC study in Caucasian men and women by A379V and G-1230A genotype. Since the 379V allele had previously shown recessive effects (Abuzeid et al., 2003), the AV and AA combined group was also compared to VV homozygotes.

Genotype associations with Lp-PLA2 activity

With regards to Lp-PLA2 activity, men homozygous for the 379V allele exhibited a non-significant trend towards higher Lp-PLA2 activity (36.9± 11.1 nmol/min/ml) compared to A379 carriers (33.3± 12.3 nmol/min/ml)($p=0.28$)(Table 4.5.4). Taking into account the G-1230A polymorphism, the G-1230 homozygous individuals did not show any difference in activity (33.3± 12.6 nmol/min/ml) compared to GA (33.9± 11.6nmol/min/ml) and AA individuals (31.95± 11.6 nmol/min/ml)($p=0.77$).

Genotype associations with oxLDL/LDL

In male and female subjects, A379V genotype was not significantly associated with oxLDL/LDL ($p=0.77$ and $p=0.18$ respectively)(Table 4.5.4). Since there was no heterogeneity of effect of gender on oxLDL/LDL ($p=0.85$), males and females were combined to examine this relationship further. In combined analysis A379V genotype was not associated with oxLDL/LDL levels ($p=0.34$) although those 379V homozygous individuals did show a trend for lower oxLDL/LDL levels (14.7± 5.7 U/mmol) compared to heterozygous (17.3± 9.5 U/mmol) and A379 homozygous individuals (17.2± 8.73 U/mmol). In both males and female subjects, G-1230A genotype was not associated with oxLDL/LDL levels (Table 4.5.5). However, when female and male subjects were considered together, those -1230 heterozygous individuals exhibited higher oxLDL/LDL levels (18.74± 10.1 U/mmol) compared to G-1230 (16.4± 7.9 U/mmol) and -1230A homozygous (14.4± 6.6 U/mmol) individuals ($p=0.01$). This result remained statistically significant after adjustment for type of diabetes, CHD status and statin use ($p=0.003$).

Genotype associations with UKPDS risk score and metabolic syndrome

Neither the A379V nor G-1230 genotypes showed significant associations with UKPDS risk score (Tables 4.5.4 and 4.5.5). There was no significant difference in A379V and G-1230A allele frequency between those with features of the MS and those without (Table 4.5.6). Similarly there was no significant difference in either A379V or G-1230A allele frequency between those with and without the presence of CHD in men and women (Table 4.5.2).

Table 4.5.4: Baseline characteristics of Caucasian subjects in the UDAC study by A379V genotype.

	Males					Females				
	AA (n=288)	AV (n=161)	VV (n=14)	p value	p value A+ / VV	AA (n=184)	AV (n=95)	VV (n=15)	p value	p value A+ / VV
Age	61.0 (13.5)	61.6 (13.9)	60.0 (15.1)	0.87	0.75	63.1 (14.2)	67.1 (12.2)	66.9 (9.0)	0.05	0.49
HDL mmol/L ‡	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)	0.99	0.91	1.5 (0.5)	1.5 (0.4)	1.4 (0.4)	0.75	0.51
LDL mmol/L +	2.6 (0.7)	2.5 (0.8)	2.8 (0.9)	0.46	0.31	2.8 (0.8)	2.7 (0.6)	2.9 (0.7)	0.39	0.78
oxLDL mU/L +	44.3 (18.4)	43.8(18.7)	49.6 (17.4)	0.68	0.39	48.0 (21.6)	45.5 (19.6)	41.6 (14.8)	0.18	0.21
oxLDL/LDL U/mmol *	17.5 (7.7)	17.3 (9.3)	15.5 (5.2)	0.77	0.49	16.7 (7.5)	17.5 (7.5)	14.0 (5.0)	0.19	0.18
%TAOS +	43.2 (13.0)	41.9 (13.9)	36.6 (16.7)	0.14	0.06	41.7 (12.7)	42.7 (13.3)	44.0 (10.3)	0.92	0.74
UKPDS risk score	31.3 (18.6)	29.9 (23.5)	29.3 (18.1)	0.76	0.77	20.7 (13.9)	20.8 (11.2)	23.1 (15.7)	0.81	0.51
Lp-PLA2 activity	33.8 (10.9)	32.4 (11.7)	36.9 (11.1)	0.25	0.28	na	na	na	na	na
nmol/min/ml *	33.3 (12.3)									

Due to the recessive nature of the A379V polymorphism, A379 allele carriers were also combined together.
*antilog of log transformed mean. SD is approximate.
+ square of square root transformed mean. SD is approximate
‡ Natural log geometric mean. SD is approximate

Table 4.5.5: Baseline characteristics of Caucasian subjects in the UDAC study by G-1230A genotype.

	Males				Females			
	GG (n=299)	GA (n=151)	AA (n=16)	p value	GG (n=185)	GA (n=105)	AA (n=8)	p value
Age	61.8 (13.0)	60.3 (14.7)	57.9 (15.6)	0.33	64.7 (13.3)	63.9 (13.7)	71.4 (11.6)	0.31
HDL mmol/L ‡	1.3 (0.4)	1.3 (0.5)	1.2 (0.4)	0.44	1.5 (0.5)	1.5 (0.5)	1.4 (0.4)	0.79
LDL mmol/L +	2.6 (0.9)	2.5 (0.9)	2.5 (0.6)	0.87	2.8 (0.9)	2.7 (0.9)	2.7 (0.5)	0.59
oxLDL mU/L +	43.6 (18.1)	46.6 (19.6)	37.2 (10.3)	0.18	46.0 (17.3)	50.2 (17.7)	33.3 (27.2)	0.18
oxLDL/LDL U/mmol *	16.7 (8.6)	19.1 (11.5)	15.8 (3.2)	0.06	16.3 (6.6)	18.6 (9.3)	13.0 (9.2)	0.07
% TAOS +	42.7 (13.2)	42.0 (13.9)	45.1 (13.7)	0.61	41.3 (13.5)	43.5 (11.5)	44.0 (12.8)	0.10
UKPDS risk score	30.2 (18.6)	32.2 (19.5)	27.2 (19.7)	0.47	20.4 (12.4)	21.2 (14.5)	24.7 (13.0)	0.63
Lp-PLA2 activity nmol/min/ml *	33.3 (12.6)	33.9 (11.6)	31.9 (11.6)	0.77	na	na	na	na

*antilog of log transformed mean. SD is approximate.
+ square of square root transformed mean. SD is approximate
‡ Natural log geometric mean. SD is approximate

Table 4.5.6: Genotype allele frequencies by MS status in Caucasian individuals taking part in the UDAC study.

	Males			Females		
	No	**Yes**	**p**	**No**	**Yes**	**p**
A379V AA	149	139		84	103	
AV	81	80		40	55	
VV	6	8		8	7	
Rare allele frequency	0.20 (0.16-0.23)	0.21 (0.17-0.25)	0.59	0.21 (0.16-0.26)	0.21 (017-0.25)	0.93
G-1230A GG	154	145		79	106	
GA	75	76		51	57	
AA	7	9		3	5	
Rare allele frequency	0.19 (0.15-0.22)	0.20 (0.17-0.24)	0.54	0.21 (016-0.26)	0.20 (0.16-0.24)	0.65

4.5.3.6 Combined genotype analysis

As discussed, the G-1230A and A379V SNPs are in strong negative LD. To investigate whether the combination of genotypes led to any additive effects, the most common combined genotype groups in Caucasian individuals were determined and are presented in table 4.5.7 (male and female). No individuals in the UDAC study were homozygous for the 379VV and -1230AA genotypes, explaining why this group was excluded from further analysis.

In combined genotype analysis G+/379VV individuals did not exhibit a significant difference in Lp-PLA2 activity (36.9± 11.8 nmol/min/ml) compared to G+/A+379 (33.4± 12.3 nmol/min/ml) and -1230AA/A+ individuals (32.0± 11.6 nmol/min/ml, p=0.50). The non-significant trend for higher activity in G+/379VV and G+/A+379 groups appeared to be largely dependent on the A379V genotype, with G-1230A exhibiting a negligible effect (Table 4.5.8). Additionally, there was no association of any of the haplotypes with UKPDS risk score or oxLDL/LDL measures (table 4.5.8).

Table 4.5.7: Combination of genotypes analysed

A379V genotype	G-1230A genotype	n total genotype
A+	G+	561
VV	G+	24
A+	AA	19
VV	AA	0

Table 4.5.8: Combined genotype associations in Caucasian individuals of UDACS

Variable	G+/A+379	G+/379VV	-1230AA/A+	p value
Lp-PLA2 activity nmol/min/ml *	33.4 (12.3)	36.9 (11.8)	31.9 (11.6)	0.50
oxLDL/LDL U/mmol *	17.3 (8.9)	14.7 (5.7)	14.4 (6.6)	0.13
UKPDS risk score	27.0 (17.7)	26.2 (16.9)	26.3 (17.3)	0.96

*antilog of log transformed mean. SD is approximate

Fig 4.5.1: Graph illustrating differences in Lp-PLA2 activity by Metabolic syndrome status in those with and without the presence of CHD(±1SD)

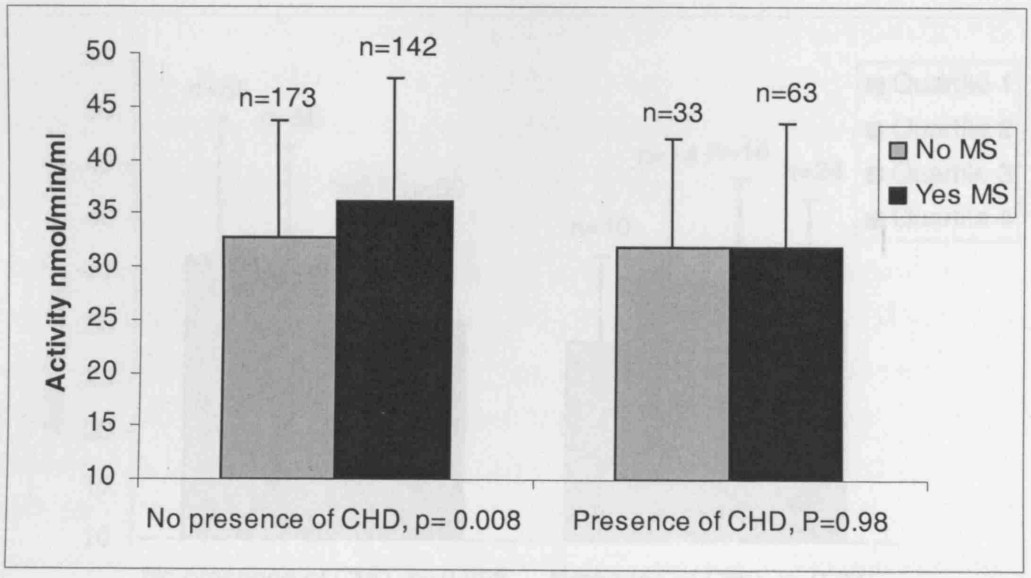


Fig. 4.5.2: Differences in Lp-PLA2 activity by quartiles of UKPDS risk score (±1SD)

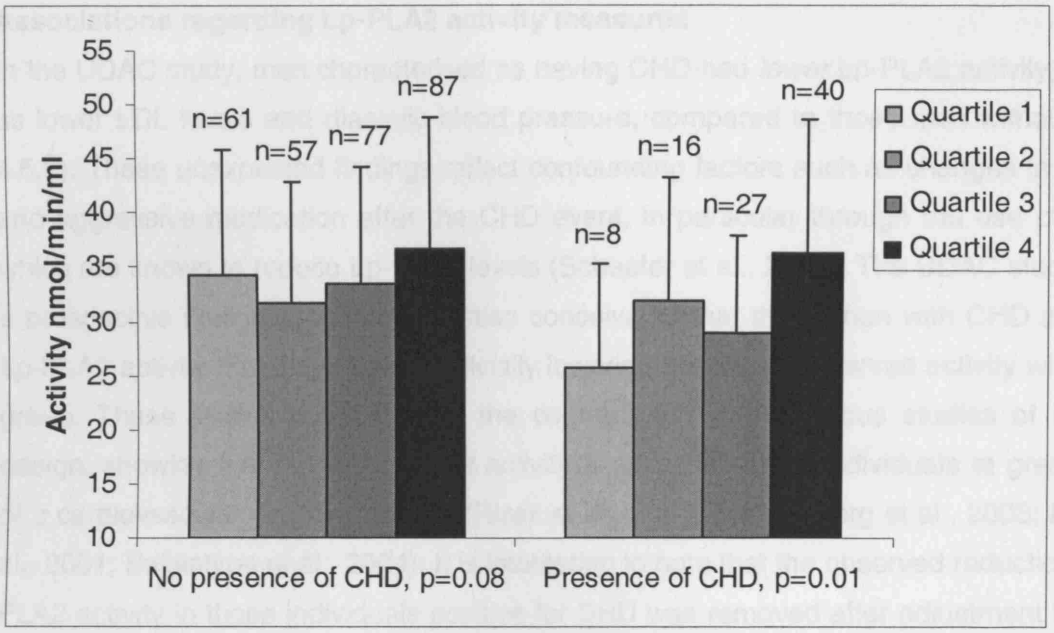
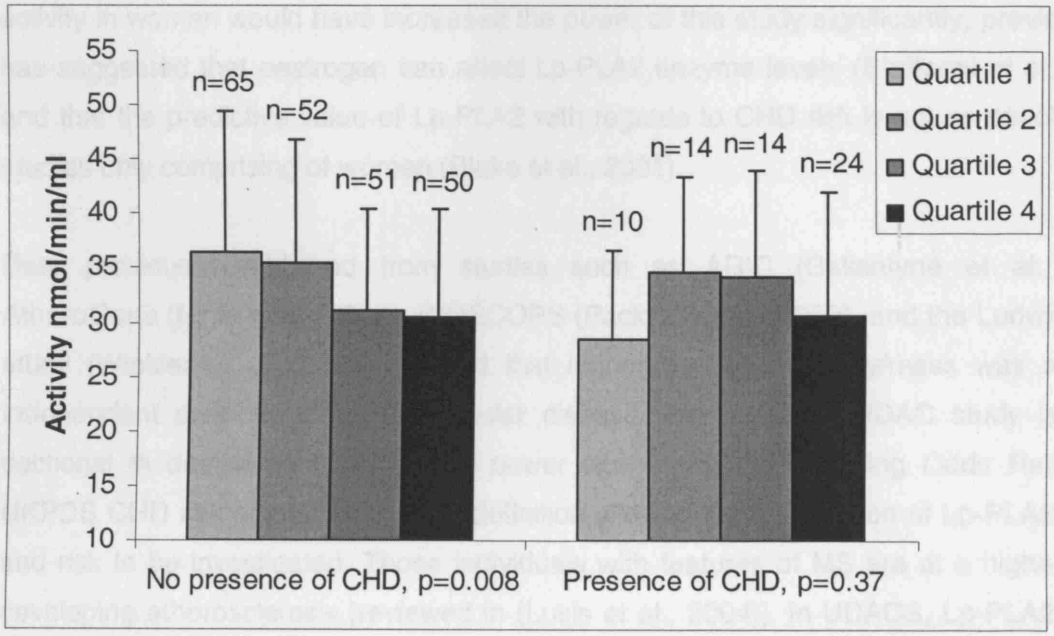


Fig. 4.5.3: Differences in Lp-PLA2 activity by quartiles of oxLDL/LDL in UDACS (±1SD)



4.5.4 Discussion

Associations regarding Lp-PLA2 activity measures

In the UDAC study, men characterised as having CHD had *lower* Lp-PLA2 activity, as well as lower LDL levels and diastolic blood pressure, compared to those men without (table 4.5.2). These unexpected findings reflect confounding factors such as changes in lifestyle and aggressive medication after the CHD event, in particular through the use of statins which are known to reduce Lp-PLA2 levels (Schaefer et al., 2005). The UDAC study is not a prospective design; it is therefore also conceivable that those men with CHD and high Lp-PLA2 activity may have died, artificially lowering the mean observed activity within this group. These factors could explain the contradiction with previous studies of differing design, showing that higher Lp-PLA2 activity is associated with individuals at greater risk of a cardiovascular event or the MS (Rizos et al., 2005; Blankenberg et al., 2003; Blake et al., 2001; Ballantyne et al., 2004). It is interesting to note that the observed reduction in Lp-PLA2 activity in those individuals positive for CHD was removed after adjustment for drug treatment and changes in lipid parameters. This would suggest that the difference in activity seen could indeed have been due to statin treatment, suggesting that this may be an efficacious method for the lowering of plasma Lp-PLA2. Although measures of Lp-PLA2 activity in women would have increased the power of this study significantly, previous data has suggested that oestrogen can affect Lp-PLA2 enzyme levels (Stafforini et al., 1997), and that the predictive value of Lp-PLA2 with regards to CHD risk is not as conclusive in studies only comprising of women (Blake et al., 2001).

Data previously published from studies such as ARIC (Ballantyne et al., 2004), Athero*Gene* (Ninio et al., 2004), WOSCOPS (Packard et al., 2000), and the Ludwigshafen study (Winkler et al., 2005) showed that higher Lp-PLA2 activity/mass was a strong independent predictor of cardiovascular disease. Although the UDAC study is cross-sectional in design and has limited power to investigate risk using Odds Ratios; the UKPDS CHD risk algorithm and MS definition allowed the association of Lp-PLA2 activity and risk to be investigated. Those individuals with features of MS are at a higher risk of developing atherosclerosis [reviewed in (Lusis et al., 2004)]. In UDACS, Lp-PLA2 activity was 7.9% significantly higher in men with the MS compared to those without, although in those patients with CHD this difference in activity was not seen, possibly due to the aggressive drug therapy and lifestyle changes implemented post- CHD event. The

significant positive association of UKPDS risk score with Lp-PLA2 activity supported the observations regarding the MS, and suggests that Lp-PLA2 activity acts as an accurate predictor or marker of CHD risk (as defined by UKPDS and MS) in diabetic subjects. In comparison to PROCAM and FRAMINGHAM risk scorings, the UKPDS risk algorithm was based upon a study of patients with diabetes (5102 patients who were followed for just over ten years)(Stevens et al., 2001) and has previously been found to be a comparable risk predictor to those scoring techniques (Song and Brown, 2004).

The association of Lp-PLA2 activity with oxidised LDL raises interesting questions about the potential pro- or anti- atherosclerotic roles of this enzyme. Higher oxidised LDL/LDL levels were significantly associated with lower Lp-PLA2 activity in the UDAC study, although this association was not apparent in those individuals with the presence of CHD, again suggesting that treatment has dampened an intermediate phenotype associated with the progression of atherosclerosis. The oxLDL/LDL measure was used to correct for differences in LDL concentration and sheds light on processes governing the rate of LDL particle oxidation (Scheffer et al., 2003). This association supports data showing that Lp-PLA2 protected LDL *in vitro* from lipid peroxidation, with the removal of this enzyme leading to enhanced oxidation as measured by conjugated dienes (Lee et al., 1999); and work by Noto *et al.* which showed in a mouse model that expression of the human Lp-PLA2 gene led to greater resistance of lipoproteins to copper mediated oxidation (Noto et al., 2003). If Lp-PLA2 is removing pro-inflammatory oxidised phospholipids and PAF from LDL, then the contradicting association between Lp-PLA2 activity and risk suggests that this is a consequence of CHD, and not causal of it. However, the hydrolysing of oxidised PC could in turn, generate high levels of Lyso-PC and free fatty acids within LDL, making the enzyme pro-atherogenic. Indeed, it has been shown that removal of Lp-PLA2 from LDL before oxidation reduces Lyso-PC content, monocyte chemo attractant properties and macrophage apoptosis of the resulting oxidised LDL (Carpenter et al., 2001; Macphee et al., 1999; Tew et al., 1996). In which case, the observed association between activity and UKDPS risk score seen here represents a causal relationship of this enzyme with atherosclerosis. Disappointingly, the results obtained regarding oxLDL/LDL levels were not mirrored by TAOS measurements. Previous associations have shown no association between plasma markers of oxidative stress and oxidised LDL (Kopprasch et al., 2002; Weinbrenner et al., 2003). The results in UDACS would therefore suggest that the role of Lp-PLA2 with regards to plasma oxidative stress is limited to its role in mediating LDL

oxidation. Unfortunately, without complex phospholipid biochemical analysis no firm conclusions concerning the causal relationship of this association can be made.

Genotype Effects

With regards to *PLA2G7* genotype, there was no significant effect of either the A379V or G-1230A polymorphism on Lp-PLA2 activity in the UDAC study. Those individuals homozygous for the 379V allele had 10.8% higher Lp-PLA2 activity compared to A379 carriers, although this difference did not reach statistical significance (p=0.28). Despite the association not reaching significance, this data supports results from the Athero*Gene* study (Ninio et al., 2004), and also data from the EPIC-Norfolk and NPHS II studies in this thesis, which showed a weak but significant association between the 379V variant and *higher* activity. However as has already been discussed in this chapter, this further complicates any understanding of the functionality of this variant. The *PLA2G7* 379V allele has been previously associated with a lower risk in the HIFMECH and Athero*Gene* studies (Abuzeid et al., 2003; Ninio et al., 2004), therefore suggesting that elevated Lp-PLA2 activity may be important in the *prevention* of atherosclerosis. However, *in vitro* (Kruse et al., 2000) the 379V Lp-PLA2 enzyme hydrolysed labelled-PAF at an increased Km and Vmax compared to the A379 form of the enzyme (although the Km and Vmax values were obtained with recombinant *E.coli* enzyme which was neither glycosylated or attached to lipoproteins) thereby suggesting that Lp-PLA2 is *pro-atherogenic*. Another complicating factor is the result obtained in this thesis from the large EPIC-Norfolk study, which did not confirm the previous association of A379V genotype with CHD risk.

The UDAC study failed to identify a statistically significant effect of A379V genotype on Lp-PLA2 activity, oxLDL/LDL levels or risk (as defined by MS and UKPDS). With contradicting data concerning the A379V polymorphism and Lp-PLA2 activity, as well as CHD risk, it is difficult to make any conclusions as to the functionality of this SNP. One possibility could be that the A379V polymorphism is part of a function altering haplotype, involving several other variants in LD with the A379V SNP. Either that or the A379V SNP is not functional at all and is in LD with another variant that does have a functional role. However, since all the studies genotyped to date were based on Caucasian subjects, haplotype structures and frequencies should be the same in these samples. In order to investigate whether the G-1230A polymorphism may have been involved in a haplotype effect, I decided to combine the A379V and G-1230A genotypes and investigate their associations. Again the results

observed suggest that the G-1230A polymorphism is having little or no effect with regards to Lp-PLA2 activity, UKPDS risk score or oxLDL/LDL measures. In individual analysis the G-1230A polymorphism was significantly associated with differences in oxLDL/LDL levels (when men and women were combined). However, the association is difficult to explain since most of the effect was seen in those heterozygous individuals and may simply be a consequence of multiple testing errors.

The UDAC study may not have been powered sufficiently to investigate all of the associations investigated. As discussed in chapter one, the metabolic syndrome is a combination of various pre-identified risk factors, with several flexible criteria depending upon the chosen definition. In addition, diabetes itself has a very broad phenotype. With regards to those continuous variables investigated, the UKPDS risk score is not ideal for investigating subtle differences in CHD risk and is more suitable as a clinician's tool. The measurement of oxidative stress by TAOS is suitable for epidemiological studies, and recent work in this laboratory has shown that TAOS tightly correlates with the gold standard measuring of F_2 iso-prostanes ($r=-0.65$, $p=0.003$, personal communication Dr Jeffrey Stephens). However, TAOS measures represent a global view of plasma oxidation, and therefore any effects on LDL oxidation by Lp-PLA2 may be lost to noise. Despite these factors, it was possible to determine an association of Lp-PLA2 activity with levels of oxLDL/LDL and the UKPDS risk score. In addition, the CHD predictive power of Lp-PLA2 was further enhanced with the association of Lp-PLA2 activity with the metabolic syndrome. It is also clear that much more detailed *in vitro* and *in vivo* work is needed to establish the precise nature of the altered enzyme kinetics of the A379V variant on Lp-PLA2 function.

Finally, there is a possibility that Lp-PLA2 could be exerting pro- and anti- atherogenic effects depending on the *in vivo* location of the enzyme. Higher levels of Lp-PLA2 activity in the plasma could prevent atherosclerosis with the increased removal of pro-inflammatory PAF, and oxidised phospholipids on the surface of LDL particles (Karasawa et al., 2003). The generation of Lyso-phospholipids and free fatty acids, although contributing to a pro-inflammatory status, may not reach concentrations in the plasma that are physiologically important. However, once in the arterial wall, Lp-PLA2 may generate a physiologically important concentration of Lyso-phospholipids and free fatty acids, altering Lp-PLA2 from a beneficial enzyme to one that is detrimental. However, until definitive

evidence about the role of the A379V variant on activity or substrate preference becomes clear, these data must be interpreted with caution.

4.5.5 Summary of UDAC study results

i) Lp-PLA2 activity was significantly associated with UKPDS risk score and those individuals with metabolic syndrome.

ii) Lp-PLA2 activity was significantly associated with the oxLDL/LDL measures, but not measures of oxidative stress.

iii) Those individuals on Statins exhibited lower mean Lp-PLA2 activity compared to those not on statin therapy.

iv) 379V individuals showed a non-significant elevation in mean Lp-PLA2 activity compared to heterozygous and A379 homozygous individuals.

v) The G-1230A polymorphism does not appear to modulate Lp-PLA2 activity in this sample.

vi) The A379V polymorphism showed no significant association with oxLDL/LDL, UKPDS risk score or metabolic syndrome.

vii) The G-1230A variant showed a borderline association with oxLDL/LDL, but was not associated with UKPDS risk score and Metabolic syndrome

viii) Combined Genotype analysis showed no associations with oxLDL/LDL, UKPDS risk score or Lp-PLA2 activity.

4.6 Association of the *PLA2G7* A379V variant with body composition changes in response to exercise training.

4.6.1 Introduction

One of the aims of this thesis was to investigate other physiological roles of Lp-PLA2 that may impact on the development of atherosclerosis, in particular, its function in the remodelling of PAF. The production of PAF is tightly regulated, balanced between a *de novo* synthesis/remodelling pathway involving the combined action of cytosolic phospholipase, A2/acyl-coenzyme A-independent transacylase, and lyso-PAF: acetyltransferase. Lp-PLA2 and other Phospholipase A2 enzyme (PLA2) family members are involved in the remodelling of platelet activating factor (PAF) to Lyso-PAF, and the consequent release of Arachidonic acid by the generation of alkyl-acyl-glycerophosphorylcholine from Lyso-PAF (Ninio, 2005; Kudo and Murakami, 2002)(Fig. 1.11). Arachidonic acid (AA) is a precursor of the eicosanoid family of potent inflammatory mediators that includes prostaglandins, thromboxanes, leukotrienes, and lipoxins. Prostaglandin I_2 (prostacyclin), which is synthesised and released from pre-adipocytes, has previously been identified as one of the main adipogenic components of serum (Massiera et al., 2003). Phospholipase A2 remodelling of PAF is a significant mechanism through which AA is liberated from phospholipids, and could therefore play a role in a diverse range of signalling pathways, one of which could lead to adipocyte differentiation and hence alterations in adipose tissue mass (Balsinde et al., 2002). In addition, Lp-PLA2 through the production of lyso-PAF and Lyso-PC, leads to the synthesis of Lysophosphatidic acid (LPA, monoacyl-sn-glycero-3-phosphate), which has been found to be abundant in mildly oxidised lipoproteins, and released from activated platelets (Moolenaar et al., 2004) (Fig. 1.11). Recent work has suggested that LPA interacts with PPARγ, which itself is known to modulate cell proliferation and adipose tissue differentiation (Zhang et al., 2004; Michalik and Wahli, 1999).

Within this thesis, the A379V polymorphism has not been found to be associated with CHD risk in the populations tested. There also appeared to be some ambiguity over the functional role of this SNP relating to enzyme activity in these studies and those published elsewhere (Kruse et al., 2000; Ninio et al., 2004). Despite extensive screening and the identification of several other variants present in the *PLA2G7* gene (Kruse et al., 2000;

Ninio et al., 2004; Stafforini, 2001), none of these SNPs exhibited a more consistent association with markers of atherosclerosis or CHD risk (Ninio et al., 2004). By contrast, the novel G-1230A polymorphism has not been found to be associated with either Lp-PLA2 activity or CHD risk in the UDAC and HIFMECH studies. However, in order to fully investigate this SNP, it was necessary to test its association with all the potential functions of Lp-PLA2. Therefore, both the A379V and G-1230A polymorphism were genotyped to investigate the mechanism by which Lp-PLA2 may affect body composition change in the Bassingbourn Army Study II, a study of young healthy Caucasian male recruits over a 10 week intensive exercise training period (Myerson et al., 2001).

4.6.2 Materials and methods

4.6.2.1 Bassingbourn Army Study II design

The study group comprised of 144 Caucasian male recruits, aged between 16-22 years, drawn from the army training regiment, Bassingbourn, UK over an 18 month period (Myerson et al., 2001). All were normotensive and free from overt cardiovascular disease, and underwent an identical 10 week intensive physical exercise programme involving mixed strength and endurance training conducted by the British Army. The study aimed to examine the effect of the Angiotensin II type I receptor antagonist, Losartan, on left ventricular growth, and to investigate the association of Angiotensin Converting Enzyme (*ACE)* gene variation with exercised- induced left ventricular growth. Recruits were genotyped for the *ACE* insertion (I)/ deletion (D) polymorphism and those homozygous for either the *ACE* I or D alleles were invited to participate in the trial, and written informed consent was obtained. Subjects were studied at the beginning and end of the training period, at which time, weight and blood pressure (mean of 3 manual measurements) were recorded. In addition to recruits undergoing cardiac magnetic resonance (CMR) to determine LV mass, forty 10mm-thick MRI images of the whole body were obtained, adipose tissue mass was quantified, and lean mass was calculated by subtracting adipose tissue mass from total body mass (Myerson et al., 2001). After baseline scans, each II or DD group was independently randomised (conforming to a prospective parallel arm double blind, randomised controlled trial protocol) to receive either 25mg Losartan or placebo daily (compliance was monitored thoroughly by officers). The Defence Medical Services College Research Committee (DMSCR) approved the human subjects protocol described.

4.6.2.2 *PLA2G7* A379V and G-1230A genotyping

Genotyping was carried out by PCR and restriction enzyme digestion as described previously in this chapter and chapter 2 (section 2.2). Table 2.1 lists the conditions used for the assays.

4.6.2.3 Statistical analysis

Genotype information was collated on an EXCEL spreadsheet. Allele frequency was determined by the gene counting method and was tested for deviation from Hardy-Weinberg equilibrium using the χ^2 test. Allelic association was considered using the statistic delta (Δ) as described previously (Chakravarti et al., 1984). Lewontin's D' was

determined from the Haploview 3.2 program developed by the Broad institute, Cambridge USA (http://www.broad.mit.edu/mpg/haploview/). The association between *PLA2G7* A379V / G-1230A genotype and continuous variables was analysed using ANOVA and linear trend on the SPSS v12.1 statistical package (Chicago, USA). All the variables analysed were normally distributed. For studying alterations in lean mass and adipose tissue, the 'percentage change' measure takes into account baseline differences in body mass, and was determined in the following way:

$$\text{Percentage change in lean or adipose tissue mass} = \frac{\text{mass, x - mass, y}}{\text{mass, y}} \times 100$$

Where x= post-training and y= pre-training.

4.6.3 Results

4.6.3.1 Baseline characteristics and allele frequencies

A379V and G-1230 genotype distribution were in Hardy-Weinberg Equilibrium with a rare allele frequency of 0.22 (95%CI 0.16-0.27) and 0.17 (95%CI 0.13-0.21) respectively. The allele frequencies were consistent with reported frequencies in European and Caucasian individuals (A379V only) (Abuzeid et al., 2003; Kruse et al., 2000; Ninio et al., 2004) as well as those found in the UDAC, EPIC and NPHS II studies. With regards to LD, the Δ between the A379V and G-1230A SNP suggested weak negative allelic association (Δ =-0.21, p=0.07) that did not reach statistical significance. By contrast D' indicated a strong allelic association between the two SNPs of 1 ($p<0.001$). The observed LD was similar to that observed for the previously genotyped studies.

There was no heterogeneity of any of the qualitative traits relating to Losartan treatment (Myerson et al., 2001), and *ACE* genotype was also found not to significantly affect lean mass and adipose tissue mass change, therefore analysis was performed on the group as a whole.

Table 4.6.1 lists the baseline characteristics of the BH2 study and table 4.6.2 shows baseline characteristics by A379V genotype. None of the traits measured differed significantly by A379V genotype at the start of the study. Table 4.6.3 lists the baseline characteristics of the BH2 study by G-1230A genotype. None of these traits significantly differed by G-1230A genotype, although there was a borderline non-significant trend for higher systolic and diastolic blood pressure in those heterozygous and -1230A homozygous individuals at baseline.

Table 4.6.1: Baseline characteristics of the Bassingbourn Army Study II. Table includes individuals who did not have end-point readings.

Trait	Numbers (n)	Mean ±1SD
Age, years	158	19.6± 2.5
BMI, kg/m^2	191	23.1± 2.4
Weight, kg	198	71.0± 9.1
Height, m	195	1.75± 0.06
Systolic BP, mmHg	176	117.3± 12.1
Diastolic BP, mmHg	176	66.9± 10.8
Lean mass, kg	165	58.8± 6.5
Adipose tissue mass, kg	167	12.1± 4.3
Left Ventricular mass, g	191	183.7± 26.2

Table 4.6.2: Baseline characteristics of the Bassignbourn Army II study by A379V genotype. Recruits would did not finish the study were still included

Trait	n	AA	AV	VV	P value	P linear trend
Age, years	151	19.6± 2.6	19.7± 2.2	19.9± 2.7	0.91	0.67
BMI, kg/ m^2	184	23.2± 2.5	22.8± 2.1	23.1± 2.5	0.63	0.44
Weight, kg	190	71.8± 9.7	69.1± 7.9	73.4± 8.0	0.16	0.44
Height, m	187	1.75± 0.07	1.74± 0.06	1.79± 0.05	0.15	0.67
Systolic BP, mmHg	168	117.5± 12.8	117.5± 11.5	114.9± 9.2	0.83	0.71
Diastolic BP, mmHg	168	67.2± 10.7	66.9± 11.2	64.9± 9.2	0.83	0.63
Lean mass, kg	159	59.3± 6.7	57.4± 6.1	60.9± 5.5	0.16	0.53
Adipose tissue mass, kg	161	12.3± 4.5	11.8± 3.4	14.1± 4.9	0.30	0.69
Left Ventricular mass, g	183	184.2± 27.2	179.7± 23.4	197.7± 28.1	0.15	0.79

Table 4.6.3: Baseline characteristics of the BH2 study by G-1230A genotype. Recruits not finishing the study were included

Trait	N	GG	GA	AA	P value	P linear trend
Age, years	152	19.6± 2.4	19.6± 2.6	20.6± 2.7	0.67	0.68
BMI, kg/ m^2	186	23.0± 2.4	23.3± 2.4	23.5± 2.8	0.76	0.46
Weight, kg	192	71.1± 8.9	70.9± 9.6	73.6± 10.6	0.73	0.73
Height, m	189	1.75± 0.06	1.74± 0.08	1.77± 0.06	0.46	0.78
Systolic BP, mmHg	170	116.3± 12.2	120.3± 11.0	122.4± 14.9	0.09	0.07
Diastolic BP, mmHg	170	66.1± 10.6	68.6± 11.0	74.0± 11.9	0.06	0.06
Lean mass, kg	161	59.1± 6.2	58.0± 7.2	61.3± 8.0	0.40	0.99
Adipose tissue mass, kg	163	12.3± 4.3	11.8± 4.2	12.4± 4.5	0.77	0.65
Left Ventricular mass, g	185	184.4± 26.7	181.9± 24.0	194.5± 25.4	0.40	0.99

4.6.3.2 Changes in measured traits by A379V genotype

Over 10 weeks of intensive physical exercise, there was no significant change in left ventricular mass (LVM), blood pressure, and BMI by A379V genotype. VV homozygotes showed a trend, although not statistically significant, towards a loss in weight whereas the AA and AV genotype groups gained weight over the ten week training period (Table 4.6.4). When the changes in body composition over the training period were examined, recruits homozygous for the V379 allele showed a significant decrease in adipose tissue mass over the training programme (-3.14± 3.0 kg) when compared to AV (-0.97± 1.8 kg) and AA (-0.82± 1.9 kg) genotype groups (p=0.01) (Table 4.6.4). There was also a borderline significant increase in lean mass in AV and VV groups over the 10 week training period (Table 4.6.4). The AV genotype group also showed a significant increase in lean mass (2.09± 2.2 kg) compared to A379 homozygotes (1.22± 1.6 kg, p=0.02) (Table 4.6.4). The change in lean muscle mass and adipose tissue were found to be tightly negatively correlated in this study ($r=-0.91$, $p<0.01$).

4.6.3.3 Corrected body composition changes by A379V genotype

When corrected for baseline differences in body size among recruits by analysing the changes in percentage adipose tissue and lean mass, individuals homozygous for the 379V allele showed a significant decrease in percentage adipose tissue (-3.61± 3.0) compared to AV (-1.67± 2.4) and AA (-1.09± 2.1) genotype groups (p=0.01 ANOVA and linear trend) (Table 4.6.4 and Fig. 4.6.1). There was also a significant increase in percentage lean mass in VV recruits (3.51± 3.1) compared to AV (1.64± 2.4) and AA individuals (1.10± 2.1, p=0.02 ANOVA and p=0.01 for linear trend) (Table 4.6.4 and Fig 4.6.1).

The design of the BH2 study meant that the selected recruits were a reasonably homogeneous population and adjustment for potential confounders such as age and systolic blood pressure, did not significantly affect the observed results (mean values remained almost constant with those un-adjusted values). ACE I/D genotype did not correlate with any measured characteristics concerning body composition, therefore the study was investigated as a whole without adjustment. Losartan treatment was randomly assigned within the study and was also found not to be associated with any changes in body composition, therefore no adjustment was applied for this potential confounder either.

4.6.3.4 G-1230A associations in the BH2 study

When changes in body composition and other traits over the ten week training period were considered, the G-1230A SNP was found to not be associated with either a change in percentage adipose tissue or percentage lean mass ($p=0.91$ and $p=0.92$ respectively), despite being in apparent tight negative LD with the A379V variant (Table 4.6.5).

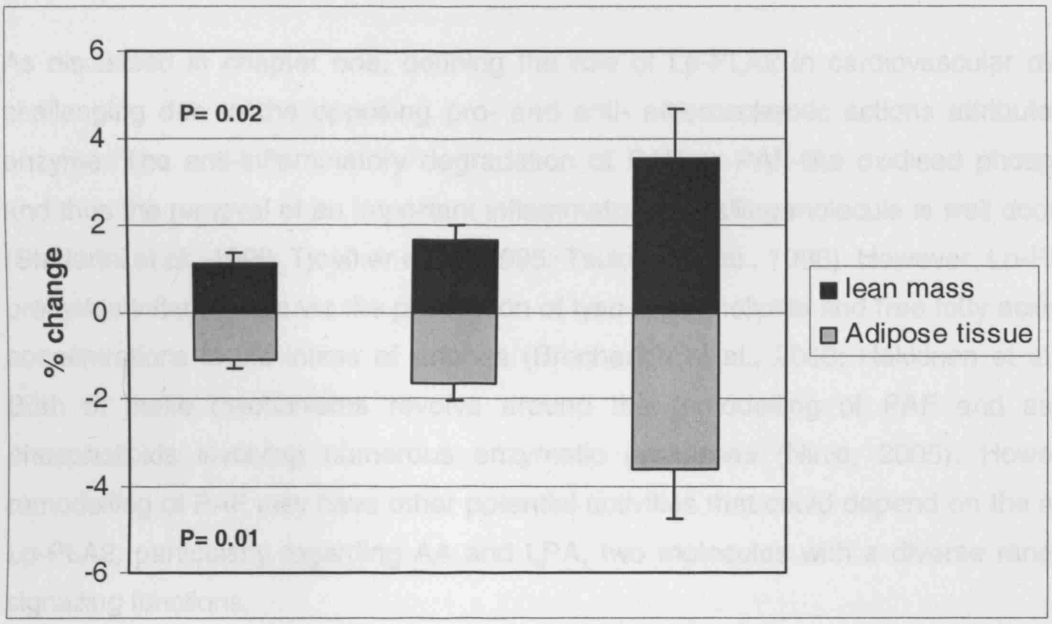
Table 4.6.4: Change in variables over the 10 week training period by A379V genotype (mean ±1SD)

Traits N=	AA [77]	AV [39]	VV [7]	*p* ANOVA	*p* linearity
Systolic BP change, mm Hg	0.90± 14.1	1.09± 12.8	-1.33± 15.2	0.92	0.86
Diastolic BP change, mm Hg	-1.14± 11.1	0.22± 12.0	1.67± 14.2	0.72	0.42
% LV mass change	4.53± 7.7	6.31± 7.6	2.73± 10.1	0.37	0.59
% BMI change	0.78± 4.0	1.53± 3.6	-1.41± 5.7	0.19	0.78
% change in weight	0.78± 4.0	1.79± 3.9	-1.40± 5.7	0.13	0.96
Lean mass change, Kg	1.22± 1.6	2.09± 2.2	1.70± 1.6	0.06	0.05
Adipose tissue mass change, Kg	-0.82± 1.9	-0.97± 1.8	-3.14± 3.0	0.01	0.03
Percentage Lean mass change, %	1.10± 2.1	1.64± 2.4	3.51± 3.1	0.02	0.01
Percentage Adipose tissue change, %	-1.09± 2.1	-1.67± 2.4	-3.61± 3.0	0.01	0.01

Table 4.6.5 : Change in variables over the 10 week training period by G-1230A genotype (mean ±1SD)

Trait N=	GG [86]	GA [30]	AA [7]	*p* ANOVA	*p* linearity
Systolic BP change, mm Hg	1.36± 13.1	0.48± 14.5	-2.71± 14.6	0.73	0.47
Diastolic BP change, mm Hg	1.18± 11.8	-1.52± 9.3	-2.43± 8.5	0.44	0.21
% LV mass change	4.70± 8.2	5.30± 7.0	4.69± 8.0	0.94	0.83
% BMI change	0.70± 4.1	1.57± 3.9	1.12± 4.2	0.60	0.41
% change in weight	0.83± 4.2	1.57± 3.9	1.11± 4.2	0.70	0.51
Lean mass change, Kg	1.46± 1.8	1.76± 2.0	1.46± 1.7	0.75	0.64
Adipose tissue mass change, Kg	-1.05± 2.2	-0.70± 1.6	-0.90± 1.8	0.83	0.61
Percentage Lean mass change, %	1.43± 2.5	1.27± 2.0	1.23± 1.7	0.92	0.70
Percentage Adipose tissue change, %	-1.45± 2.5	-1.27± 2.0	-1.19± 1.8	0.91	0.67

Fig. 4.6.1: Change in percentage lean mass and adipose tissue over 10 weeks training (±1SD)



4.6.4 Discussion

As discussed in chapter one, defining the role of Lp-PLA2 in cardiovascular disease is challenging due to the opposing pro- and anti- atherosclerotic actions attributed to the enzyme. The anti-inflammatory degradation of PAF or PAF-like oxidised phospholipids, and thus the removal of an important inflammatory signalling molecule is well documented (Stafforini et al., 1996; Tjoelker et al., 1995; Tsukioka et al., 1996). However, Lp-PLA2 also promotes inflammation via the production of lyso-phospholipids and free fatty acids at high concentrations in the intima of arteries (Brocheriou et al., 2000; Hakkinen et al., 1999). Both of these mechanisms revolve around the remodelling of PAF and associated phospholipids involving numerous enzymatic processes (Ninio, 2005). However, the remodelling of PAF may have other potential activities that could depend on the activity of Lp-PLA2, particularly regarding AA and LPA, two molecules with a diverse range of cell signalling functions.

The aim of this study was to investigate the potential association of the newly identified G-1230A and previously described A379V genetic variants in the *PLA2G7* gene with changes in body composition, specifically lean mass and adipose tissue. The BH2 follow-up study was originally intended to investigate changes in left ventricular heart muscle mass by Losartan treatment and ACE I/D genotype before and after 10 weeks of intensive physical training, but also includes detailed measures of the whole body lean and adipose tissue mass.

Although there was no significant effect of A379V genotype on overall change in weight and BMI over the ten week training period, there was a strong association between A379V genotype and changes in body composition. V379 homozygotes were associated with a significant *lowering* in percentage adipose tissue mass, whilst also associating with a significant *increase* in percentage lean mass compared to AV and AA individuals. The significant linear trend with AV recruits exhibiting intermediate values, suggests that even though the numbers of 379V homozygotes were small, the conclusions were still valid. By way of contrast, the G-1230A variant showed no association with any of the traits investigated in the study, despite being in strong LD with the A379V variant. The negative LD between these two SNPs means that although the 379V allele was found to be

associated with changes in body composition, this would not be observed in the common G-1230 allele due to the presence of other confounding genotypes.

Unfortunately, the functional effect of the A379V variant has yet to be determined fully, with contrasting results reported in this thesis and elsewhere (Kruse et al., 2000; Ninio et al., 2004). However, it is possible to speculate on the modulating role that Lp-PLA2 might play with regards to changes in adipose tissue and lean muscle mass. In a sepsis model it has been shown that serum Lp-PLA2 levels correlate significantly with several cascade substances downstream of AA production, such as leukotriene B4 and thromboxane B2 (Takakuwa et al., 1994). In addition, recently published data has demonstrated that PAF initiates the release of AA in a murine macrophage cell line, potentially via the PAF receptor and eventual activation of cytosolic PLA2 (Schaloske et al., 2005). The generation of AA and eicosanoids may affect adipose tissue differentiation by the activation of peroxisome proliferator-activated receptors (PPAR), which mediate the effects of fatty acids and their derivatives at the transcriptional level. There is evidence that long chain fatty acids such as AA and a number of derivatives, are ligands for PPARγ (Forman et al., 1997; Yu et al., 1995; Krey et al., 1997; Kliewer et al., 1997), a critical regulator of adipocyte differentiation and function (Michalik and Wahli, 1999). Whilst the precise mechanism of PPARγ activation by this pathway is under debate, it is thought that prostacyclin binds with its cell surface receptor, activating the protein kinase A pathway (Vassaux et al., 1992), and up-regulating the early expression of two transcription factor CCAAT-enhancer binding proteins (C/EBPδ and C/EBPβ) (Belmonte et al., 2001). Secondly, there is evidence that prostacyclin binds directly to PPARδ (Forman et al., 1997). C/EBPδ, C/EBPβ, and PPARδ then act together in pre-adipocytes to up regulate the critical expression of PPARγ, leading to adipogenesis (Massiera et al., 2003).

Higher Lp-PLA2 activity has previously been found to be associated with obese, diabetic individuals (Kudolo et al., 1997). Despite confusion over the functional effect of the A379V variant, genetic variation in the Lp-PLA2 enzyme could influence adipositiy. A possible mechanism for the effects seen here is that the A379V variant is associated with differences in Lp-PLA2 activity (Kruse et al., 2000; Ninio et al., 2004), which could in turn, affect the hydrolysis of PAF to Lyso-PAF, eventually altering the concentration of AA and its derivatives through this remodeling pathway (Ninio, 2005). Variation in AA could affect PPARγ activation and pre-adipocyte differentiation, and thus fat mass. This effect may be

amplified by the fact that intense exercise can induce an inflammatory response (Montgomery et al., 1996), with Lp-PLA2 and prostaglandins being known to be elevated in these circumstances (Demers et al., 1981; Memon et al., 1999). The related secretory PLA2 IIA has also been shown to be correlated with body mass index (Boekholdt et al., 2005b), and the release of AA upon hydrolysis of phospholipids (Mounier et al., 2004), supporting the mechanism by which PLA2 enzymes affect body composition through this remodelling pathway.

Apart from the synthesis of AA, another important cell signaling molecule produced by the remodeling pathway and potentially implicated in the modulation of adipocyte differentiation is that of LPA. Lp-PLA2 and other secretory PLA2 enzymes are able to hydrolyse oxidised acyl chain phospholipids, generating Lyso-PC (or Lyso-PAF depending on the substrate, Fig. 1.11). Lyso-PC and Lyso-PAF are substrates for Lysophospholipase D leading to the generation of LPA (Ninio, 2005). Lysophosphatidic acid acts through its specific G protein-coupled receptors which trigger proliferation, migration, and survival in of a variety of cells; in particular, LPA is a potent inducer of smooth muscle cell de-differentiation (Hayashi et al., 2001). LPA is also synthesised at the extracellular face of adipocytes by a secreted lysophospholipase D (Simon et al., 2005). Conflicting data suggests that LPA may inhibit and initiate adipocyte differentiation: LPA present in conditioned media increases the growth of preadipose cell lines in culture through the activation of MAPK (Pages et al., 2000), however, recent *in vitro* work suggests that LPA exhibits an inhibitory effect on the development of adipose tissue, through the inhibition of PPARγ expression (Simon et al., 2005). Variation in the activity of Lp-PLA2 (associated with the A379V variant) could therefore influence this pathway as well, explaining some of the effects seen regarding adipose tissue mass changes in the BH2 study.

The change in percentage lean mass observed is more difficult to fully explain with our current understanding of Lp-PLA2. Metabolites from the generation of AA regulate muscle-protein turnover, in particular elevated levels of Prostaglandin E2 have been implicated in cancer cachexia (Baracos, 2000). Cytosolic PLA2 has also been identified as a negative modulator of striated muscle growth. *PLA2G4A* -/- mice have exaggerated skeletal and cardiac muscle growth, corrected by the addition of AA (Haq et al., 2003). Although neither of these processes directly implicates the Lp-PLA2 enzyme. It is possible that the change

in lean mass observed is simply a reflection of the adipose tissue mass lost, since lean mass is calculated by subtracting adipose tissue mass from total mass.

In conclusion, *PLA2G7* A379V genotype was found to be associated with body composition changes in healthy young males exposed to prolonged rigorous exercise. The small sample size of this study has meant that the VV homozygous group comprises only seven individuals, although the design of shared environment, identical training, and accurate phenotypic measurement at baseline and follow-up increases the power of the study considerably. In addition, no measures of Lp-PLA2 activity, AA or LPA were available to assess whether any of the proposed mechanisms are related to the activity and function of Lp-PLA2. Despite these limitations, the results from BH2 do at least propose a novel role for Lp-PLA2 in the development of obesity, and by its association, cardio-vascular disease.

As a postscript to these conclusions, a larger study of over 400 recruits has recently been set up by clinical fellows based in the cardiovascular genetics group at UCL (Big Heart 4, BH4), with a very similar study design and recruitment process. In addition, none of the recruits in this larger study were selected by ACE I/D genotype, and were not part of a Losartan-placebo trial. Genotyping for the *PLA2G7* A379V variant in this population was subsequently undertaken in March 2005. Unfortunately, adipose tissue and lean mass measurements have yet to be fully analysed by project collaborators, and problems remain over the study database. The BH4 study would have had greater statistical power to test the associations reported in this thesis, but unfortunately until these issues have been resolved, analysis of this study is not possible.

4.6.5 Summary of BH2 results

i) 379VV individuals exhibit a marked decrease in adipose tissue over ten weeks of intensive training, compared to AV and AA genotypes.

ii) By contrast, AV and VV individuals show a significant increase in lean muscle mass compared to AA homozygotes.

iii) Novel G-1230A polymorphism showed no association with any measured traits within the study

iv) Lp-PLA2 may modulate body composition changes over a ten week intensive training period.

4.7 In conclusion

Chapter 4 of this thesis has tried to establish the causal relationship of the Lp-PLA2 enzyme with atherosclerosis. While the UDAC and BH2 studies provided novel associations of *PLA2G7* A379V genotype with intermediate phenotypes such as oxLDL/LDL, the MS, and body composition; a major aim of this study was to address whether the Lp-PLA2 enzyme was causal with regards to CHD or acting as a marker of disease. The EPIC-Norfolk study provided continued evidence of an association between Lp-PLA2 enzyme activity and CHD risk (those results relating Lp-PLA2 enzyme activity to UKPDS risk score in UDACS also appeared to confirm this), although adjustment for confounding factors, in particular lipid measures, led to this association becoming non-significant. In the NPHS II and EPIC-Norfolk studies a significant association of *PLA2G7* A379V genotype with Lp-PLA2 activity was seen (similar to that seen in the AtheroGene study (Ninio et al., 2004)), although this association contradicted previous *in vitro* data (Kruse et al., 2000). Neither the putatively functional A379V variant nor the G-1230A novel promoter SNP were found to be associated with CHD risk in any of the studies investigated.

The contradictory data in this thesis made it difficult to ascertain whether the Lp-PLA2 enzyme is directly involved in the progression of atherosclerosis. Apart from problems concerning the functional effect of the A379V variant on Lp-PLA2 enzyme activity, confounding factors that might affect the association observed between Lp-PLA2 activity and CHD risk also presented significant challenges in answering this question. If one considers the relationship of Lp-PLA2 activity with CHD risk on its own then three possible explanations exist to explain this relationship. Firstly, another factor such as LDL could be the real explanation of the association seen between CHD risk and Lp-PLA2 activity in the EPIC-Norfolk study (confounding)(Fig. 4.7.1 A). Indeed, adjustment for LDL in the EPIC-Norfolk study led to Lp-PLA2 activity no longer being associated with CHD risk; a scenario that could be explained by confounding. The second potential scenario is an adaptive response to disease: atheroma could result in the elevation of Lp-PLA2 activity in response to inflammatory cytokines. Lp-PLA2 itself may have no effect on disease in this situation (reverse-causation)(Fig. 4.7.1 B). The third possibility is that Lp-PLA2 itself is causally linked for the reasons highlighted in the introduction of this thesis (Fig. 4.7.1 C).

One way of testing to see which of these three scenarios exists would require a specific inhibitor of the Lp-PLA2 enzyme and a subsequent randomised drug-placebo trial. As long as the inhibitor of interest shows no pleiotropic effects (as in the case of statins) any reduction in CHD risk associated with the taking of the medication could be assigned to the effects on the Lp-PLA2 enzyme. Indeed, recent interest has revolved around specific inhibitors of Lp-PLA2 and their potential impact on atherosclerosis, although only preliminary results are currently available (Blackie et al., 2002; Blackie et al., 2003). The second method uses the concept of mendelian randomisation (Minelli et al., 2004; Thomas and Conti, 2004). Genotype is not subject to confounding (a particular problem when investigating Lp-PLA2) since it is determined at conception by the random inheritance of one of each parental allele. A test of causality could be achieved by studying the relationship between CHD risk and a genetic determinant of an intermediate phenotype. In the case of Lp-PLA2, enzyme activity has been shown to be significantly associated with CHD risk. And A379V genotype was found to be associated with CHD risk in the HIFMECH and AtheroGene studies (Abuzeid et al., 2003; Ninio et al., 2004). Assuming A379V genotype has a functional effect on enzyme activity; the consistency between the expected CHD risk from phenotype-disease and genotype-disease can therefore be tested (Casas et al., 2005). If the indirect genetic estimate of CHD risk were smaller (Fig. 4.7.2, Arrow B) than the directly observed association of Lp-PLA2 activity with CHD risk (Fig. 4.7.2 Arrow A), then the analysis would suggest that the directly observed association of Lp-PLA2 activity with CHD risk was affected by residual confounding and/or reverse causations, leading to the conclusion that Lp-PLA2 is unlikely to be causal in the development of atherosclerosis (Hingorani and Humphries, 2005).

This appears to be a rational method by which to test the associations found in this thesis and in other published studies [reviewed in (Sudhir, 2005)]. By conducting a meta-analysis of data it may be possible to more accurately confirm the relationship of Lp-PLA2 enzyme with CHD. However, several critical assumptions remain: firstly, that the A379V genotype acts only by altering the plasma activity of Lp-PLA2 and not other functional effects of the enzyme. Secondly, the differences in Lp-PLA2 activity by A379V genotype are small, requiring very large studies to remove imprecision of the un-confounded genetic estimates of the effect of Lp-PLA2 on CHD risk (Hingorani and Humphries, 2005). Results from this thesis would suggest that these two assumptions need to be investigated thoroughly before conducting a mendelian randomisation approach using the A379V variant. Despite

these concerns, the addition of the studies genotyped in this chapter should provide a significant platform for using this type of genetic approach in the future.

Fig. 4.7.1: The three potential relationships of Lp-PLA2 activity with CHD. A) Confounding. B) Reverse causation. C) Causal relationship.

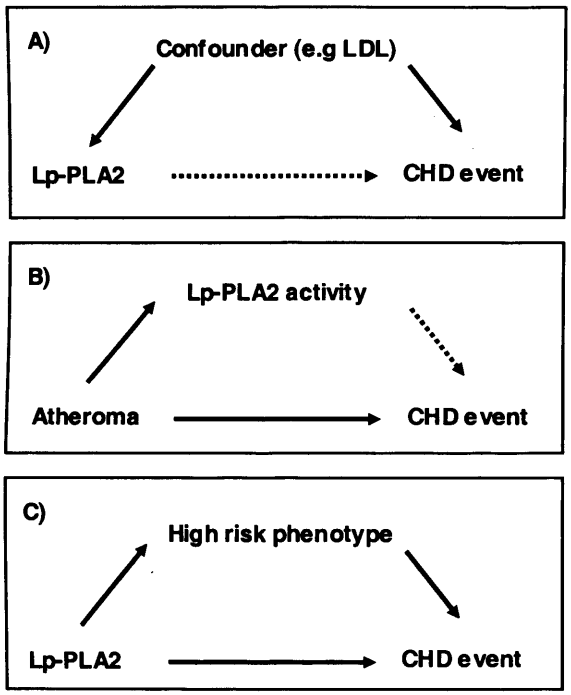
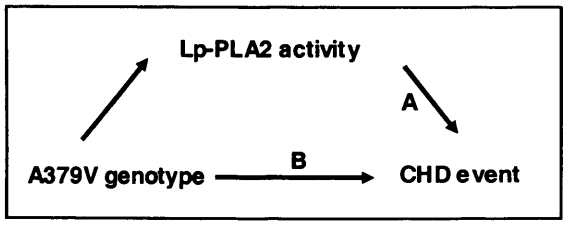


Fig. 4.7.1: A test of causality.



CHAPTER 5

INVESTIGATION OF NOVEL *PLA2G7* PROMOTER POLYMORPHISMS AND THEIR EFFECT ON TRANSCRIPTIONAL REGULATION

5.1 Introduction

The epidemiological associations of the novel G-1230A SNP with markers of atherosclerosis, Lp-PLA2 activity, and CHD risk have been discussed in the previous chapter of this thesis. Unfortunately, the G-1230A promoter polymorphism was not found to be significantly associated with either CHD risk (HIFMECH) or markers implicated in the progression of atherosclerosis (UDACS and BH2). However, this does not completely rule out the possibility of this SNP being functional. Many variants exhibit moderate to low effects which may not reach statistical significance due to confounding factors such as; allelic heterogeneity, confounding from population structure or inadequate sample sizes (Colhoun et al., 2003; Farrall and Morris, 2005; Suh and Vijg, 2005).

An added complication when ascertaining the functionality of a SNP in association studies is the LD that exists between variants. Some of the trends noted in this thesis regarding the G-1230A SNP could reflect those associations found with the putatively functional A379V variant or another, as yet unidentified, functional variant. Previous analysis of the AtheroGene study tried to determine which of the common putatively functional SNPs in the *PLA2G7* gene were independently associated with Lp-PLA2 enzyme activity and CHD risk (Ninio et al., 2004). Haplotype analysis of these SNPs suggested that the main effects seen were the result of the A379V variant. However, this analysis only concentrated on two SNPs in the proximal promoter region: a T to C change at position -403 (5' of the transcription start site) and a C to G change at position -209. Other variants in the promoter were not considered in the analysis and this work was unable to conclusively prove the functionality of the A379V variant. In order to fully define the functional nature of any variant requires the development of an assay independent of the confounding factors found in association studies.

Investigating the precise functional effect of the A379V variant is difficult because of the complicated nature of predicting protein structure and accurately assaying function. However, determining the functional contribution of promoter polymorphisms is currently a more achievable objective. One can determine how well a specific proximal promoter fragment is driving expression of a gene by cloning the promoter fragment of interest into the multiple cloning site (MCS) of the pGL-3 Basic plasmid vector. The pGL3-basic vector contains the firefly luciferase gene, and expression of this gene is essentially controlled by

the promoter fragment ligated 5' of the gene. Cultured cells are then transiently transfected with the pGL3-Basic vector containing the construct, leading to the expression of the luciferase gene. The relative level of expression of the luciferase gene is measured by the addition of a specific substrate which the expressed luciferase enzyme hydrolyses, resulting in the release of a luminescent signal that can be accurately quantified. Alterations to the promoter construct (caused by polymorphisms in this case) and their effect on the rate of transcription can therefore be accurately assessed. The accuracy of this system has been aided by the development of the dual-luciferase assay by Promega Ltd (Southampton, UK)(Sherf et al., 1996). In parallel to transfecting cells with the pGL3-Basic vector (containing the construct of interest), a second vector is also introduced into the cell. In the case of the Promega assay this vector contains the Renilla gene (which utilises a different substrate and emits light at an altered wavelength to that of the Luciferase gene) driven by a suitable viral promoter. Using a luminometer it is possible to measure luminescence from the two vectors with the Renilla vector acting as an endogenous control. This in turn enables accurate quantification of gene expression correcting for any variability in transfection efficiencies between experiments. This technology has been successfully used to investigate the *PLA2G7* promoter region. By deleting sections of the proximal promoter Cao *et al.* were able to demonstrate that a short 72bp 5'-flanking region was sufficient for 65% of basal *PLA2G7* promoter activity, and that there was more than one area in the 1.3kb 5' genomic sequence conferring promoter activity (see Fig. 1.9 in chapter 1)(Cao et al., 1998). Recently, our laboratory has used the luciferase reporter assay to investigate the effect of promoter polymorphisms in the hormone sensitive lipase gene and the *APOA5* gene (Talmud et al., 1998; Talmud et al., 2005).

The successful use of the luciferase assay is dependent upon the 'host' cell line used for transfection. Chosen cell lines should try and represent as close as possible the tissue source of the enzyme *in vivo*, since elements that control transcription may differ among cell types. Prior research has shown that Lp-PLA2 is secreted into the plasma by platelets, neutrophils, macrophages (expression levels are elevated upon monocyte differentiation (Elstad et al., 1989)), T-lymphocytes (Tjoelker and Stafforini, 2000), and tissue sources that are consistent with the haematopoietic origin of the enzyme, such as lymph nodes, the thyroid gland (Cao et al., 1998; Tjoelker et al., 1995), and liver (Howard et al., 1997).

These physiological observations were taken into account when choosing a suitable immortalised cell line for transfection of the *PLA2G7* promoter.

As already discussed, the *PLA2G7* promoter has previously been screened using an SSCP based approach, with two common variants being found (T-403C and C-209G)(Ninio et al., 2004). Chapter 3 of this thesis also used an SSCP approach to scan the *PLA2G7* promoter region, and successfully identified the G-1230A polymorphism. In order to determine the functional effect of this novel promoter polymorphism, a 1.9kb of the *PLA2G7* promoter would be introduced into the pGL3-Basic plasmid 5' of the firefly luciferase gene. These constructs would be altered by site-directed mutagenesis (SDM) to include a wild-type background of the two previously identified T-403C and C-209G SNPs. Alterations to the G-1230A position by SDM would then enable the independent effect of this variant to be examined. Transfection of pGL3-Basic plasmid constructs would be carried out in a monocyte/macrophage-like cell line (HL-60) and a hepatocyte cell line (Huh-7), both of which are cells grown in culture that represent similar cellular sources found in humans. HL-60 cells have previously been identified as secreting Lp-PLA2 when differentiated into macrophages (Narahara et al., 1993), and there is some evidence that HepG2 cells (a similar hepatocyte cell line to Huh-7 cells) also express Lp-PLA2 (Satoh et al., 1993; Satoh et al., 1991). By measuring the relative level of firefly luciferase activity for each construct, a comparison could be made regarding the effect of the G-1230A SNP on *PLA2G7* promoter activity, and fully confirm the functional role of this SNP.

5.2 Materials and methods

5.2.1 PCR amplification and ligation into pGL3-Basic

A detailed description of the PCR amplification and ligation of the 1.9kb section of *PLA2G7* promoter DNA can be found in section 2.4.1. In brief, 1.9kb of the *PLA2G7* promoter was amplified from genomic DNA using a pair of oligonucleotides that contained two restriction endonuclease recognition sites [Forward *(Mlu I)* 5'-CG**ACGCGT**TGCTGGCAATGAGAGAAGTG-3' and Reverse *(Bgl II)* 5'-GA**AGATCT**TCCGCCTCAAAAGAAAGAAA-3']. The PCR was modified in order to achieve a high fidelity amplification of the sequence: proof reading Platinum *Pfx* DNA polymerase (Invitrogen, Paisley UK) was used according to the manufacturers' instructions. The reverse oligonucleotide was designed to be 300bp 3' of the *PLA2G7* transcription start site. This was to ensure that the short proximal promoter region essential for promoter activity was included (Cao et al., 1998). Previous work suggested that the amplified promoter fragment does not include any strong silencer elements (Cao et al., 1998).

PCR product was run on a 1% agarose gel in order to confirm the correct size of the fragment. The band of interest was cut out, and purified using the GFX Gel band purification kit according to manufacturers' instructions (Amersham Biosciences Ltd., Buckinghamshire UK). Both the pGL3-basic (Promega Ltd, Southampton UK) and PCR fragment were then incubated with Mlu I and Bgl II restriction endonucleases in order open up the plasmid and generate overlapping 'sticky' ends on the PCR product and plasmid. Ligation of the vector and PCR fragment was performed by mixing 100ng of vector and 15ng digested PCR product with 1μL ligase and 1μL ligase buffer (New Englannd Biolabs, Hertfordshire UK). Using the pre-designed oligonucleotides that flank the MCS, it was possible to sequence the ligated plasmid to ensure that the promoter was in the correct orientation (see section 2.5 for sequencing conditions and protocol).

5.2.2 Site-directed mutagenesis (SDM) and sequencing

In order to generate large quantities of plasmid, *E. coli* DH5α™ competent cells (Invitrogen, Paisley UK) were transformed by heat shock, as described in section 2.4.2. Selection of successfully transformed colonies is possible since the pGL3-Basic vector contains a gene encoding ampicillin resistence (see Fig 5.2). By plating on ampicillin agar it was possible to pick colonies that had successfully taken up the pGL3-Basic vector, and

grow them in 5ml LB broth containing ampicillin. Plasmid DNA was initially extracted using the QIAprep spin miniprep kit (Qiagen Crawley, UK) according to manufacturers instructions described in section 2.4.3. Previous data from the GeneCanvas website (www.genecanvas.org) suggested that the -403 and -209 variants were in complete positive LD with each other when genotyped in the SIPLAC study (D'= 0.99, p<0.0005). Similar results were also obtained in the Athero*Gene* study (Ninio et al., 2004). In order to investigate the G-1230A variant's effect on promoter activity, a wild-type (WT) background of T-403 and C-209 genotype was introduced into the promoter sequence using SDM. Although the LD between the G-1230A SNP and the two other promoter SNPs is unknown, by selecting the WT background it was possible to ensure that only luciferase activity differences corresponding to the G-1230A variant would be measured. Site directed mutagenesis (SDM) was used to introduce point mutations, and performed using the Quikchange-SDM kit supplied by Stratagene (California, USA) according to section 2.4.4. Table 5.1 lists the SDM oligonucleotides used to introduce the necessary changes to the G-1230A and C-209G positions. The PCR product used for cloning contained the initial genotype -1230A, T-403 and -209G. Therefore two sets of oligonucleotides were required to change the -209 position to C-209, and the -1230A to a G-1230 (it was unnecessary to change the T-403C position). All alterations to the sequence were confirmed by sequencing described in section 2.5, and re-transformed into *E. coli* DH5α™ competent cells (Invitrogen, Paisley UK). Point mutations occurring in the firefly luciferase gene in the pGL3-Basic plasmid could lead to an altered luciferase enzyme activity. To avoid this, all promoter fragments were cut out of the pGL3-Basic vector using *Mlu I* and *Bgl II*, and re-ligated into a new pGL3-Basic vector. As with the previous ligation steps, the construct was then sequenced to ensure that the promoter was ligated in the proper orientation.

Once the cloned promoter fragments were successfully altered to include each -1230 genotype, the Gene Elute HP maxiprep kit (Sigma-Aldrich, Poole UK) was used to generate high quantities of plasmid DNA for transfection experiments from 150ml bacterial cultures (see section 2.4.3). Maxiprep plasmid was ethanol precipitated and re-constituted in 500μL of sterilised Sigma water. Although the *Taq* polymerase used in cloning the *PLA2G7* fragment is a proof-reading enzyme, errors can still occur in the sequence. To ensure that all the constructs were correct, each cloned promoter (maxiprep) was fully

sequenced using internal and external primers (see section 2.5 and Table 2.6 list full details of oligonucleotides used in sequencing).

Table 5.1: SDM oligonucleotides used to introduce changes in sequence. Base pairs in bold represent the introduced point mutation

Allele introduced	Forward and Reverse Oligonucleotide
G-1230	FOR 5'-TCTTAAGGTCAAAAGAAGAC**GT**TAGAGATGTCTTTGGTAGGG
	REV 5'-CCCTACCAAAGACATCTCTAA**C**GTCTTCTTTTGACCTTAAGA
C-209	FOR 5'-CAGGCATTGCCTGGCTCT**C**TCCGCGGCGGGCTAAG
	REV 5'-CTTAGCCCGCCGCGGA**G**AGAGCCAGGCAATGCCTG

5.2.3 Transfection of the Huh-7 cell line

Stable culturing of Huh-7 cells is described in section 2.7.2 of the materials and methods section. All cells used for transfection were between the 34th and 36th passage. Since no prior research has established whether the *PLA2G7* gene is expressed in Huh-7 cells, cultured cells were initially tested for *PLA2G7* mRNA. Three wells of Huh-7 cells cultured in a 96 well plate for 24 hours were taken and assayed for *PLA2G7* gene expression. Lysis, mRNA extraction, cDNA synthesis and RT-PCR for the *PLA2G7* gene is described fully in sections 2.6 and 2.8.1 of chapter 2 and also chapter 6. For a negative control, three blank wells were 'sham' lysed with lysis buffer and mRNA extracted using an identical method to that described above.

The lipofectamine™ 2000 kit (Invitrogen Ltd. Paisley, UK) was used to successfully transfect Huh-7 cells according to the protocol in section 2.8.2.2 of chapter 2. After 48 hours the transfection was tested according to the instructions given in section 2.8.2.3. The layout for transfection for the Huh-7 experiment was as follows: Each column of a 96 well plate was dedicated to an individual construct (Fig. 5.1). Each construct containing the *PLA2G7* promoter was co-transfected alongside the Renilla-TK (pRL-TK) vector (Fig. 5.2); this enables the firefly luciferase value for each transfection to be adjusted for transfection efficiency (pRL-TK acts as an endogenous control). One column for each experiment was transfected with an empty pGL3-Basic vector (same vector as used for cloning the *PLA2G7* promoter) and all luciferase values were adjusted for this background reading in the subsequent analysis. As a positive transfection control, the pGL3-Control vector was

used (Fig. 5.2) which contains a powerful SV-40 virus promoter 5' of the luciferase gene and a 3' SV-40 enhancer element. If transfection was successful then the pGL3-control vector would give a strong positive luminescent signal. A negative control transfected with only pUC 19 plasmid (no luciferase gene is coded in this plasmid as shown in Fig. 5.2) was also included.

The activities of the firefly and renilla luciferases were measured sequentially from a single well using a luminometer (PerSeptive Biosystems CytoFluor Series 4000 fluorescence multi-well plate reader). Full details of all running conditions can be found in section 2.8.2.3. The firefly luciferase reporter was measured first by adding Luciferase Assay Reagent II (LAR II) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction was initiated simultaneously by adding 'Stop and Glo®' reagent to the same tube. The Stop and Glo® reagent also produces a 'glow-type' signal from the Renilla luciferase, which decays slowly over the course of the measurement. All measurements were then automatically logged onto an EXCEL spreadsheet.

5.2.4 Transfection of HL-60 cell line

Stable culturing of the HL-60 cell line is described in section 2.7.3 of the materials and methods chapter and was carried out in 6 and 12-well flat bottomed dishes. Assaying of the *PLA2G7* promoter activity is also described in detail in section 2.8.2 of the materials and methods chapter. All transfection attempts were on cells growing between the 5^{th} and 10^{th} passage. Prior to transfection, cells were treated with 20nM PMA in order to differentiate the monocytes into macrophages which then adhere to the plate surface. Previous research has demonstrated that differentiation of these cells leads to an increased secretion of Lp-PLA2 (Narahara et al., 1993). The Effectene® Transfection Kit (Qiagen, Crawley UK) was used to transfect all constructs. The Effectene® kit is specifically designed for transfecting primary cell cultures and monocyte cell lines. After 48 hours the transfection was tested according to the instructions given in section 2.8.2.3.

5.2.5 Statistical analysis

All data was entered into an EXCEL spreadsheet before being copied into an SPSS document. All statistical analysis was done by myself using SPSS v12.0.1. Each construct was transfected in a single column of a 96 well plate, creating 8 individual experiments (for 8 wells, see Fig. 5.1). For each 96 well plate run, the two constructs were transfected, along with a pGL3-Basic empty vector, positive control (pGL3-control) and negative control (pUC 19) (Fig. 5.1). For each individual construct transfected, a firefly luciferase luminescent score (from the pGL3-Basic vector) was taken along with a luminescent score for the pRL-TK endogenous control. A normalised ratio which takes into account different transfection efficiencies between wells was therefore achieved by dividing the firefly luciferase luminescence by the Renilla luminescence for each well. This normalised ratio for the eight wells was then averaged to give a mean ratio for each transfected construct. The mean of each construct was then normalised against the background reading of the pGL3-Basic construct by dividing the mean ratio for each construct by the mean ratio observed for the pGL3-Basic construct. The entire experiment including positive and negative controls was repeated 6 times. Individual comparisons between promoter constructs were analysed using an independent sample T-test. P values of <0.05 were considered significant.

Fig 5.1: Layout of a 96 well plate for two transfection experiments. pGL3-Basic is used to normalise for background luciferase activity. pGL3-Control is used to confirm successful transfection.

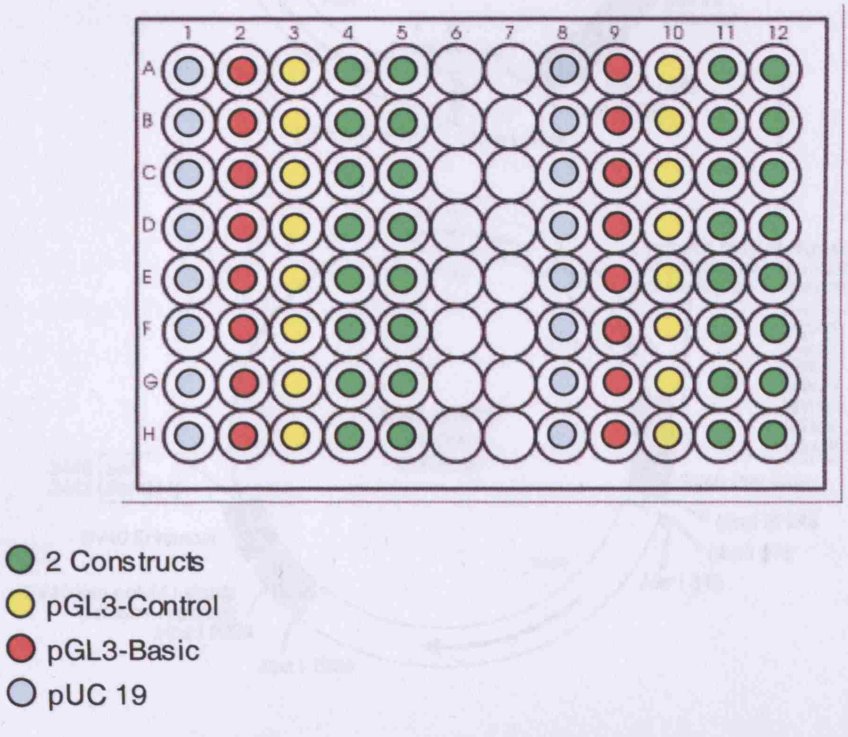
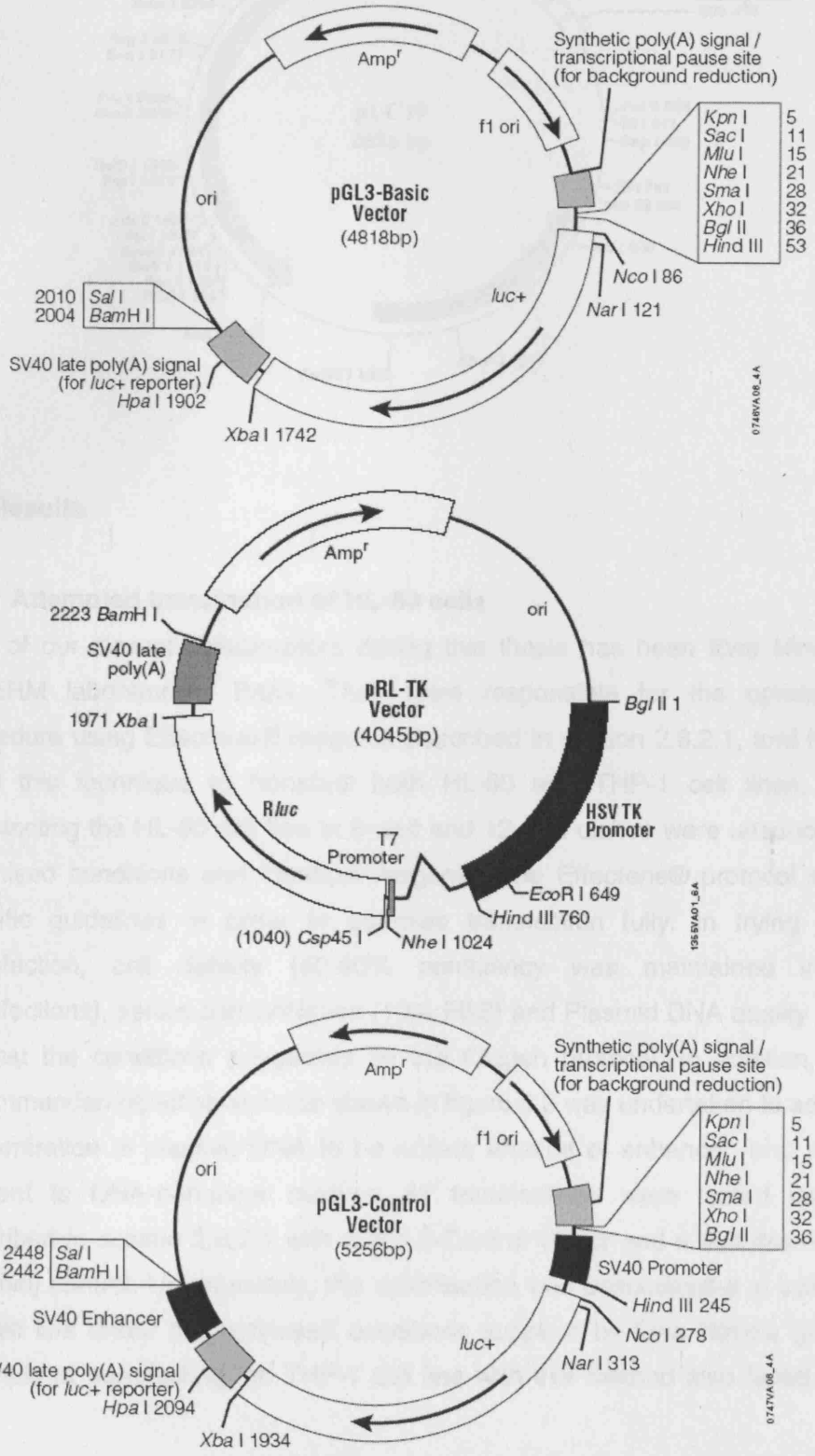
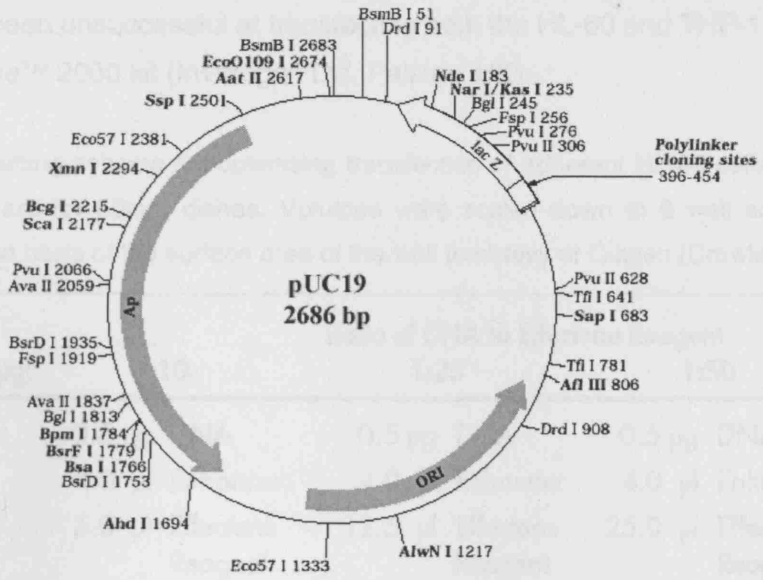


Fig 5.2: Schematic diagrams of the pGL3-Basic, pGL3-Control, pRL-TK and pUC19 vectors (Courtesy of Promega, Southampton UK)





5.3 Results

5.3.1 Attempted transfection of HL-60 cells

One of our closest collaborators during this thesis has been Ewa Ninio's group at the INSERM laboratories, Paris. They were responsible for the optimised transfection procedure using Effectene® reagents described in section 2.8.2.1, and have successfully used this technique to transfect both HL-60 and THP-1 cell lines. Initial efforts at transfecting the HL-60 cell line in 6 well and 12 well dishes were unsuccessful using their optimised conditions and identical reagents. The Effectene® protocol supplies a set of specific guidelines in order to optimise transfection fully. In trying to optimise this transfection, cell density (40-80% confluency was maintained in all attempted transfections), serum concentration (10% FBS) and Plasmid DNA quality were all checked against the conditions suggested by the Qiagen protocol. In addition, a manufacturer recommended pipetting scheme shown in figure 5.3 was undertaken to achieve the correct concentration of plasmid DNA to be added, amount of enhancer, and ratio of Effectene reagent to DNA-enhancer mixture. All transfections were tested using the method described in section 2.8.2.1 with a pGL3-Control vector and a suitable negative (pUC 19 plasmid) control. Unfortunately, this optimisation was unsuccessful at transfecting the HL-60 cell line under the optimised conditions supplied by Ewa Ninio's group. In addition, attempts at transfecting the THP-1 cell line with this method also failed. Colleagues had

previously been unsuccessful at transfecting both the HL-60 and THP-1 cell lines using the lipofectamine™ 2000 kit (Invitrogen Ltd. Paisley, UK).

Fig. 5.3: Pipetting scheme for optimising transfection of adherent HL-60 cells. Volumes shown here are for 60mm dishes. Volumes were scaled down to 6 well and 12 well formats on the basis of the surface area of the well (courtesy of Qiagen (Crawley, UK))

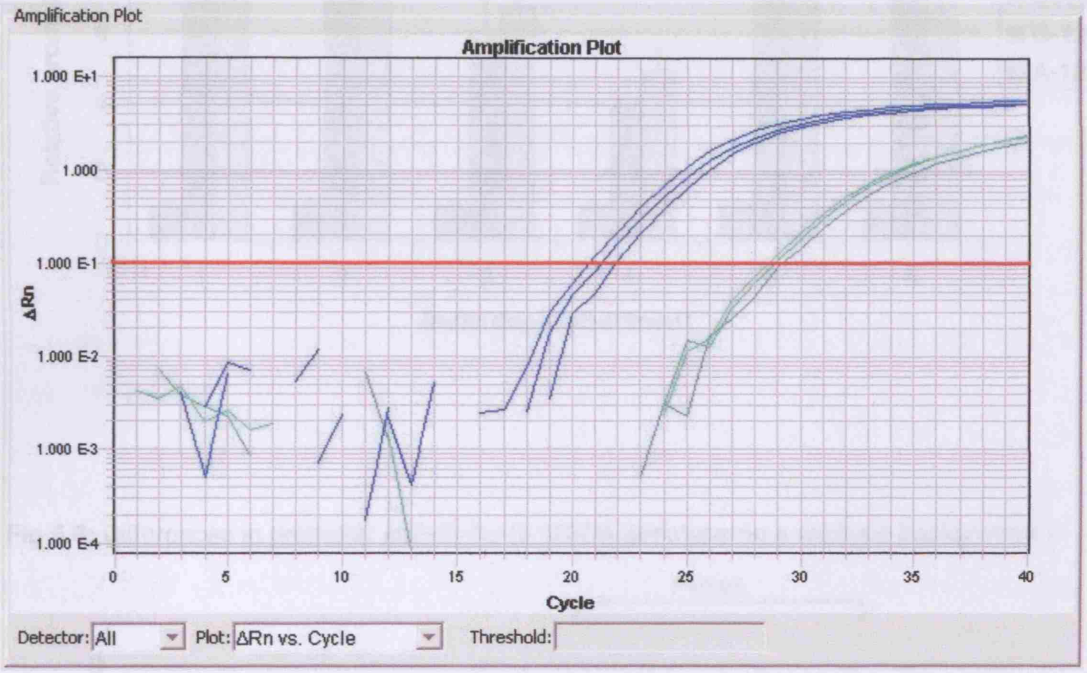
	Ratio of DNA to Effectene Reagent		
DNA (µg)	1:10	1:25	1:50
0.5	0.5 µg DNA 4.0 µl Enhancer 5.0 µl Effectene Reagent	0.5 µg DNA 4.0 µl Enhancer 12.5 µl Effectene Reagent	0.5 µg DNA 4.0 µl Enhancer 25.0 µl Effectene Reagent
1.0	1 µg DNA 8 µl Enhancer 10 µl Effectene Reagent	1 µg DNA 8 µl Enhancer 25 µl Effectene Reagent	1 µg DNA 8 µl Enhancer 50 µl Effectene Reagent
2.0	2 µg DNA 16 µl Enhancer 20 µl Effectene Reagent	2 µg DNA 16 µl Enhancer 50 µl Effectene Reagent	2 µg DNA 16 µl Enhancer 100 µl Effectene Reagent

5.3.2 *PLA2G7* mRNA expression in Huh-7 cell line

Unfortunately the failure to transfect the HL-60 cell line meant that other cell systems needed to be considered. The Huh-7 cell line has been successfully used in this laboratory to determine the functionality of common SNPs present in the *APOA5* gene (Talmud et al., 2005). Huh-7 cells originate from human liver cells and are similar in morphology to HepG2 cells which have been shown to secrete Lp-PLA2 (Satoh et al., 1991). A two step RT-PCR using the Taqman system and specific *PLA2G7* probes (described in greater detail in chapter 6) was used to determine if Huh-7 cells express the *PLA2G7* gene. Figure 5.4 shows the amplification plot of the three individual test experiments. The blue traces show that the Taqman machine successfully amplified *PLA2G7* cDNA in all three cell experiments, therefore confirming mRNA expression of the *PLA2G7* gene in Huh-7 cells. The second set of green lines represents the expression of a control gene (Ubiquitin C)

that should be stably expressed in most cell types. Negative controls of just passive lysis buffer failed to show a reading.

Fig 5.4: RT-PCR amplification plot of the three Huh-7 cultured wells. Blue lines represent *PLA2G7* gene expression, and green lines represent corresponding Ubiquitin C expression. Unfortunately SDS software does not show negative controls graphically.



5.3.3 Relative promoter activity of the G-1230A variants

Six individual transfection experiments were run for the two different constructs. The mean intra-assay CV for the pGL3-Basic vector for all six experiments was 7.6%. Figure 5.5 shows the differences in the relative promoter activity of the G-1230 and -1230A constructs after normalisation for transfection efficiency (the endogenous control, pRL-TK) and pGL3-Basic readings. Although there appears to be differences in reporter assay activity in individual experiments, when the mean values of all six experiments were considered (Figure 5.6) there was no significant difference in promoter activity between the G-1230 (6.85± 1.61) and -1230A (6.91± 1.27) construct (p=0.94).

Fig 5.5: Differences in promoter activity between all 6 transfection experiments.

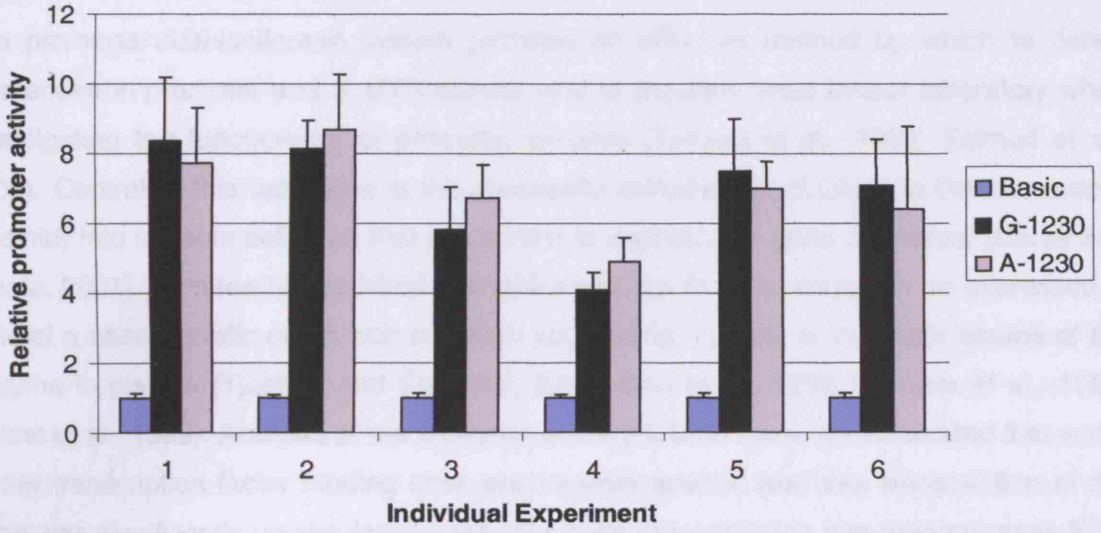
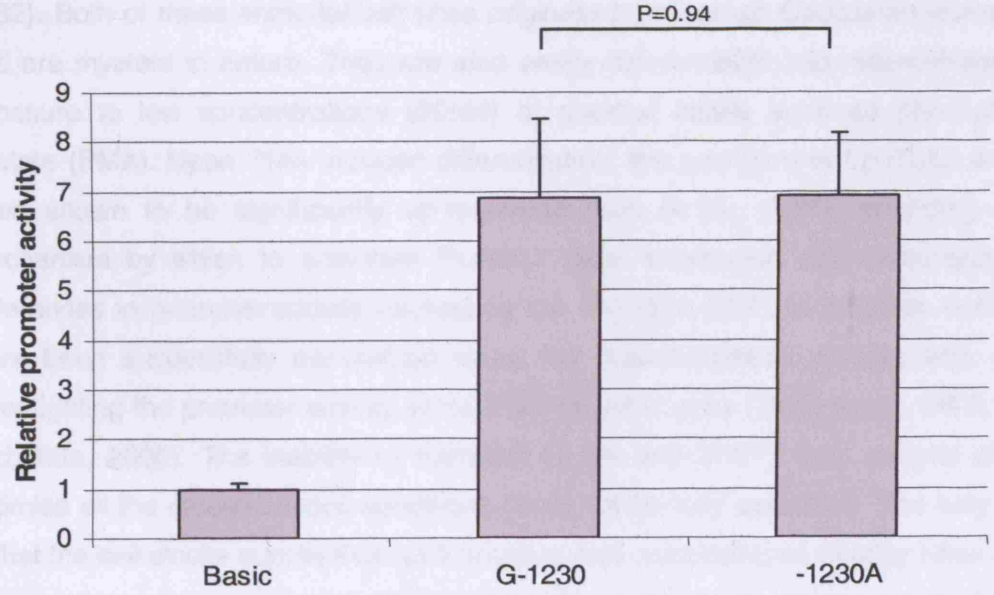


Fig 5.6: Differences in promoter activity by G-1230A genotype on a wildtype background



5.4 Discussion

The promega dual-luciferase system provides an effective method by which to detect differences in promoter and 3' UTR activity, and is regularly used by our laboratory when investigating the functionality of promoter variants (Talmud et al., 1998; Talmud et al., 2005). Central to this technique is the successful introduction of DNA (in this scenario a plasmid) into suitable cell lines that are known to express the gene of interest (Carey and Smale, 2001). As already described in chapter one, Lp-PLA2 is known to be expressed in cells of a haemopoietic origin with research suggesting that this is the major source of the enzyme in plasma (Tjoelker and Stafforini, 2000; Cao et al., 1998; Tjoelker et al., 1995; Asano et al., 1999). Analysis of the promoter of the *PLA2G7* gene demonstrated that some of the transcription factor binding sites are myeloid specific and that transcription of the gene was significantly up-regulated upon monocyte differentiation into macrophages (Cao et al., 1998; Elstad et al., 1989).

Several immortalised cell lines with similar properties to human monocytes have been developed, in particular THP-1 and HL-60 cell lines (Collins et al., 1977; Tsuchiya et al., 1982). Both of these immortal cell lines originate from human Caucasian leukaemia cells and are myeloid in nature. They are also easily differentiable into macrophages through exposure to low concentrations (20nM) of phorbol esters such as phorbol myristate acetate (PMA). Upon PMA induced differentiation, the secretion of Lp-PLA2 enzyme has been shown to be significantly up-regulated (Lee et al., 1994), providing a suitable mechanism by which to stimulate *PLA2G7* gene expression and investigate potential differences in promoter activity caused by the G-1230A SNP. In addition, both cell lines have been successfully transfected using the dual luciferase system, with one study investigating the promoter activity of the PAF receptor gene (Pang et al., 1995; Ogretmen and Safa, 2000). The inability to transfect HL-60 and THP-1 cells despite an effort to optimise all the recommended conditions could not be fully explained. The only possibility is that the cell stocks supplied to our laboratory had compromised viability when compared to our collaborators', since two different lipid-based delivery techniques (see chapter 2 for a description of Lipofectamine 2000™ and Effectene™ methods) failed to transfect these cell types properly. Failure to transfect these types of cells meant that the human hepatocellular carcinoma derived cell line, Huh-7 was used, since there is evidence that the liver is a source of Lp-PLA2 in humans (Howard et al., 1997). Indeed, the related

HepG2 cell line has been shown to express the Lp-PLA2 protein (Satoh et al., 1993; Satoh et al., 1991). An RT-PCR for this experiment confirmed that the *PLA2G7* gene is expressed in the Huh-7 cell line. However, contradictory data concerning the *in vitro* and *in vivo* regulation of *PLA2G7* expression through modulators of inflammation such as; bacterial LPS, TNF-α, IL-1 and IL-8 (discussed in section 1.4.2.2) meant that the experiments for this thesis were conducted in the absence of any potential inflammatory stimulus.

When considering differences in luciferase reporter activity for the G-1230A variant, no significant difference in promoter activity between the two constructs was observed ($p=0.94$). Unfortunately this experiment only investigated basal differences in promoter activity. Despite this, the data presented here supports the bio-informatic analysis conducted in chapter 3 which demonstrated that the G-1230A variant did not lie within or disrupt any particular transcription factor binding site, which in turn may have conferred a functional effect on the SNP. Subsequent analysis in association studies also suggested that this SNP was not associated with markers of atherosclerosis or CHD risk. Indeed, the non-significant trends seen in these association studies probably reflected the strong LD between the G-1230A and A379V variants. However, as discussed in chapter 4, no conclusions could be made about the functionality of this variant without an accurate functional assay being developed. The principle of direct gene candidate association studies is reliant on only analysing those SNPs that are predicted to be putatively functional. However, this method is often constrained from a practical point of view since limited public efforts have been made to comprehensively determine functional variants within genes, along with a limited ability to *a priori* predict SNPs with functional consequences (Suh and Vijg, 2005). With this in mind, it was important to fully determine the functional effect of the G-1230A in parallel to the genotyping of relevant association studies investigating CHD risk, Lp-PLA2 activity and other relevant traits.

Despite the failure to detect significant differences in promoter activity in this transfection experiment, a functional role for the G-1230A SNP cannot be completely excluded. While the dual-luciferase system represents a sensitive functional assay by which to detect relative differences in promoter activity (Sherf et al., 1996), the detection limit of the assay itself may prevent any subtle differences in activity being observed. In addition, the assay is not representative of an *in vivo* model: the lack of any inflammatory stimuli in this

experiment prevented the observation of differences in *PLA2G7*-driven promoter expression caused by the interaction of the G-1230A SNP with an as-yet undefined inflammatory response-element binding-site. An additional problem is that promoters themselves are complex systems reliant on multiple protein-protein and protein-DNA interactions in order to regulate transcription (Remenyi et al., 2004). By excising only a proportion of the promoter there is the danger that important transcription factors are left out, with adverse affects on any subsequent transfection experiment. Fortunately, Cao *et al.* have shown through the use of deletion constructs that a 72bp section 5' of the *PLA2G7* transcription start site is responsible for 65% of *PLA2G7* promoter activity, while there appeared to be no difference in promoter activity between a construct extending -637bp and another -3416bp 5' of the transcription start site (Cao et al., 1998). The 1.9kb section of promoter used for this thesis incorporated the minimal 72bp and -637bp promoter region and was extended to include all the proximal transcription factor binding sites previously identified by Cao *et al.* (Cao et al., 1998). This hopefully reduced the possibility of important transcription factor binding sites being excluded.

Apart from the G-1230A SNP, several other promoter variants have been identified in the promoter region of the *PLA2G7* gene. In particular, Ninio *et al.* identified the relatively frequent T-403C and C-209G variants (Ninio et al., 2004). Both of these promoter SNPs failed to show an independent association with CHD risk in the AtheroGene study (Ninio et al., 2004), and investigation via the TESS transcription binding factor website (Schug and Overton, 1997) suggested that neither of these variants affected any putative transcription factor binding sites. However, both variants lie within the proximal promoter region as defined by Cao *et al.* and could be exhibiting moderate effects on transcription efficiency, that in combination with other variants may significantly alter *PLA2G7* transcription (Cao et al., 1998). In order to determine the specific contribution of the G-1230A SNP, all constructs were examined on a wild-type background of the T-403 and C-209 alleles. However, it was the intention of the author to investigate all the identified promoter variants separately as well as investigating the three SNPs in different haplotype combinations. Unfortunately, upon re-sequencing of the constructs inconsistencies caused by unsuccessful site-directed mutagenesis were observed. Initial sequencing of the constructs prior to transfection did not show these inconsistencies, and the reasons for this are unclear since both sets of sequence (before and after transfection experiments) were taken from the same maxiprep DNA. Therefore it has not been possible to investigate

whether the T-403C and C-209G are potentially functional or if all three SNPs operate in a haplotype. To address this issue, work will continue after the submission of my thesis.

In conclusion, the analysis presented here suggests that the G-1230A SNP does not represent a functional variant with regards to transcription of the *PLA2G7* gene. Chapter 4 of this thesis demonstrated that the G-1230A SNP is neither associated with CHD risk nor Lp-PLA2 modifiable traits. As such, this variant does not appear to represent a suitable SNP for further investigation in direct gene candidate association studies. However, other variants identified in the proximal promoter region such as the T-403C and C-209G variants are still to be investigated with regards to their functionality and do warrant further investigation.

5.5 Summary of results

i) THP-1 and HL-60 cell lines failed to transfect successfully using Effectene™ and Lipofectamine 2000™ transfection kits

ii) A 1.9kb section of *PLA2G7* promoter sequence was successfully introduced into the pGL3-Basic vector. Two constructs containing each combination of G-1230A SNP were introduced into the sequence using site-directed mutagenesis

iii) Taqman two step RT-PCR showed that Huh-7 cells (liver) express *PLA2G7* mRNA. These cells were then transfected with the *PLA2G7* promoter constructs and the pRL-TK endogenous control vector using Lipofectamine 2000™

iv) Under basal conditions there were no significant differences in luminescence between the G-1230 and -1230A alleles.

CHAPTER 6

ALTERATIONS IN *PLA2G7* GENE EXPRESSION IN RESPONSE TO SIMVASTATIN TREATMENT

6.1 Introduction

Statins have been widely acknowledged as safe and effective drugs that reduce LDL cholesterol in plasma with parallel reductions in triglyceride and a modest rise in HDL [reviewed in (Wierzbicki, 2004)]. Recent meta-analysis of over 90,000 participants in 14 randomised trials has also shown statins to be very effective at reducing the 5 year incidence of major coronary events, irrespective of the individual's initial lipid profile (Baigent et al., 2005). Statins themselves are inhibitors of a key intracellular enzyme involved in the generation of cholesterol, HMG-CoA reductase (Fig. 6.1). Inhibition of this enzyme prevents the conversion of Acetoacetyl-CoA and Acetyl-CoA into Mevalonate, which is a precursor to cholesterol production (Schonbeck and Libby, 2004; Wierzbicki, 2004). However, recent attention has focused on other potential effects of statin therapy that may be beneficial in the prevention of atherosclerosis. Indeed, the Heart Protection Study (HPS) suggested that treatment effects in certain individuals might not depend solely on LDL lowering, since certain subjects with low levels of LDL still found benefit in taking a statin (Heart protection Study Collaborative group, 2002). Recent attention has focused on some of the effects of statins on inflammatory pathways previously identified as being important in the progression of atherosclerosis (Schonbeck and Libby, 2004).

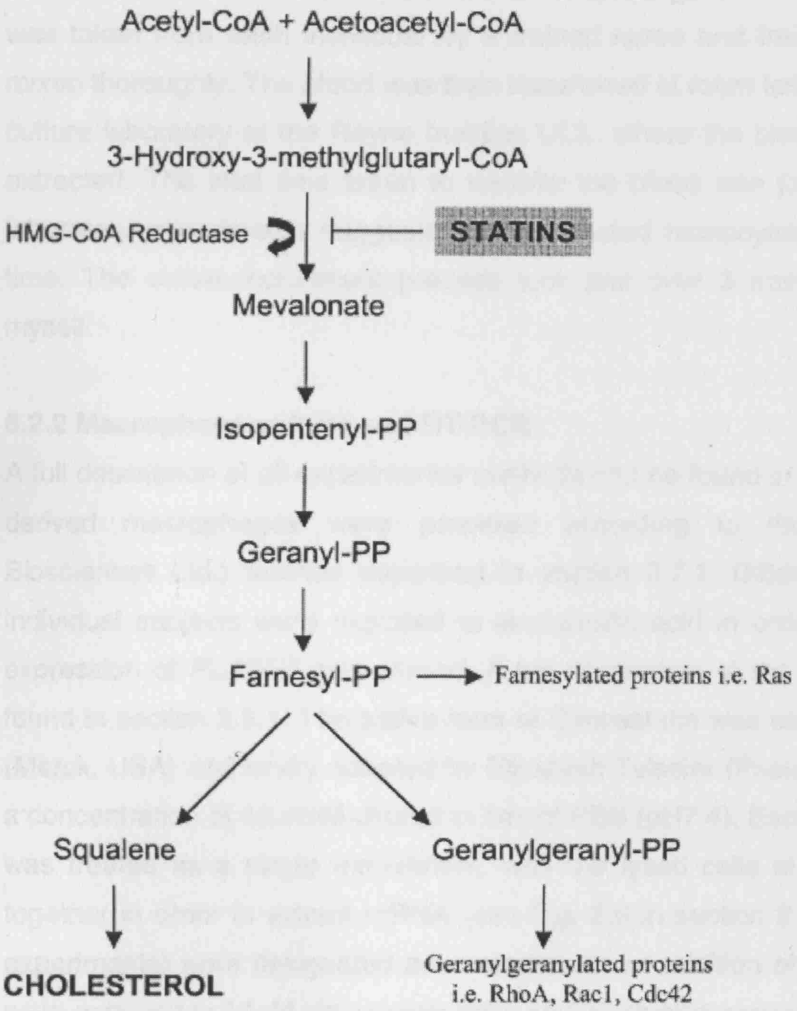
A particularly interesting association seen in the UDAC study analysis of chapter 4 was the association of Lp-PLA2 activity with statin treatment. Those individuals on statins had significantly lower measures of Lp-PLA2 activity ($p=0.04$) compared to those individuals not on medication. This result was not totally unexpected since several epidemiological studies have shown that Lp-PLA2 mass levels and activity are suppressed in those individuals receiving statin treatment. Atorvastatin for example has been shown to lower Lp-PLA2 levels by 24% (compared to a drop of 45% in LDL cholesterol) in those patients with CHD, receiving 40mg/day of statin over a 36 week period (Schaefer et al., 2005). Whilst in another randomised placebo trial, 80mg Fluvastatin treatment lowered Lp-PLA2 activity by 23% while decreasing small dense LDL subfractions by 29% in T2DM individuals (Winkler et al., 2004). Work conducted by Tsimihodimos et al. went further and found that in patients with dyslipidaemia, Atorvastatin preferentially reduced Lp-PLA2 activity associated with the densest LDL sub-fractions (Tsimihodimos et al., 2002).

This reduction in Lp-PLA2 activity/mass could simply have been the result of a reduction in LDL cholesterol levels. However, to date there has been no investigation into whether *PLA2G7* gene expression is affected directly by the presence of statin. As well as reducing LDL-cholesterol levels in plasma (and elevating HDL levels)(Wierzbicki, 2004), statins cause a reduction in intracellular cholesterol which can in turn have several effects, one of which is on transcription factors that are mediated by intracellular cholesterol levels. Sterol regulatory element binding proteins (SREBP) are particularly susceptible to these alterations in intra-cellular cholesterol, and mediate the expression of several proteins involved in cholesterol and fatty acid metabolism (de Nigris et al., 2002). Some of the pleiotropic effects seen from statin therapy may be partly a result of SREBP transcription binding domains being present in promoter regions of several genes (Liao, 2003). There is also increasing evidence that statins exhibit direct anti-inflammatory properties via the inhibition of pro-inflammatory cytokines and chemokines, as well as adhesion molecule expression on the surface of leukocytes (Ridker et al., 1998; Steffens and Mach, 2004).

Analysis of the proximal (1kb 5' of the transcription initiation start site) promoter of the *PLA2G7* gene has not revealed an SREBP or related transcription binding factor site (Cao et al., 1998). Despite this, Tsimihodimos et al. demonstrated that low doses of Atorvastatin led to an increase in Lp-PLA2 activity measured in the media of cultured macrophages, although this result was not replicated at higher doses above 50nmol/L, and there was no apparent difference in *PLA2G7* mRNA levels between treated and untreated cells (Tsimihodimos et al., 2002). As discussed in chapter 1, Lp-PLA2 production is mediated by cells of a haemopoietic origin and particularly differentiated macrophages (Elstad et al., 1989). With this in mind, I decided to investigate whether the reduction in Lp-PLA2 activity and mass seen in UDACS and other drug-placebo trials was not only due to a reduction in LDL levels but also due to a reduction in *PLA2G7* gene expression. Monocytes would be extracted from healthy middle aged individuals (none of whom were on statin medication) taking part in the NPHS II prospective study and differentiated *in vitro* into mature macrophages. These would then be exposed to simvastatic acid (the active compound of Simvastatin) at varying concentrations and mRNA would be extracted. In order to quantify the relative expression of the *PLA2G7* gene, two step 'real time' PCR was used. The first step involves the synthesis of cDNA from mRNA template extracted from treated macrophages. The second step involves 'real time' PCR using the Taqman system® which is able to detect, by the use of fluorescent probes, the exponential increase in PCR

product with each cycle. By using theses techniques, the direct effect of statin on *PLA2G7* gene expression, independent of LDL levels, could be investigated.

Fig. 6.1: Schematic of the pathway inhibited by statins.



6.2 Materials and methods

6.2.1 Study Sample and blood collection

Seven healthy middle-aged men from the NPHS II study were selected at random by our Statistician, Jackie Cooper and recruited at the North Mimms general practice. Three additional volunteers were also recruited from the British Heart Foundation laboratories at UCL (all fitted the desired clinical characteristics). All were free from any clinically apparent

infection, inflammation and CHD at the time of venous sampling. The volunteers were pre-selected homozygous for the A379V variant (5 AA379, and 5 379VV), and on the basis that none had been treated with a statin. Ethical approval was obtained for this study from the UCL/UCLH ethics committee and all subjects gave informed consent. 40ml of blood was taken from each individual by a trained nurse and transferred to EDTA tubes and mixed thoroughly. The blood was then transferred at room temperature by car to the tissue culture laboratory at the Rayne building UCL, where the blood was spun and monocytes extracted. The total time taken to transfer the blood was just over one hour, and initial laboratory experiments suggested that extracted monocytes were fully viable after this time. The entire recruitment process took just over 3 months and was completed by myself.

6.2.2 Macrophage culture and RT-PCR

A full description of all experimental methods can be found in chapter 2. In brief, monocyte derived macrophages were prepared according to the Ficoll-paque (Amersham Biosciences Ltd.) method described in section 2.7.1. Differentiated macrophages from individual subjects were exposed to simvastatic acid in order to determine whether the expression of *PLA2G7* was altered. A full description of the experimental design can be found in section 2.8.1. The active form of Simvastatin was extracted from a Zocor® tablet (Merck, USA) and kindly donated by Elisabeth Teissier (Pasteur Institute- Lille, France) at a concentration of 43.4mM diluted in 1ml of PBS (pH7.4). Each column of the 96 well plate was treated as a single experiment, with the lysed cells of each column being pooled together in order to extract mRNA (see Fig. 2.9 in section 2.8.1). 3 columns (3 separate experiments) were designated as controls with no addition of simvastatic acid, 3 columns were exposed to 10μM simvastatic acid, and 3 columns exposed to 25μM simvastatic acid. Twenty four hours before treatment, the medium (macrophage serum free media, MSFM) was removed and the cells washed with 200μL of 1×PBS solution. The PBS was carefully removed and 200μL of medium supplemented with 5% FBS was added to each well of the 96 well plate. On the day of treatment 3 volumes of medium were made up accordingly:

Control: 9.3μL of 1×PBS (pH7.4) diluted into 8ml of MSFM (5%FBS)

10μM: 1.86μL of simvastatic acid and 7.44μL 1×PBS diluted in 8ml MSFM

25μM: 4.65μL of simvastatic acid and 4.65μL of 1×PBS diluted in 8ml MSFM

The medium was removed carefully and the cells washed in 200μL of 1×PBS. The PBS was then removed and 200μL of the treated/control medium was added to each well using

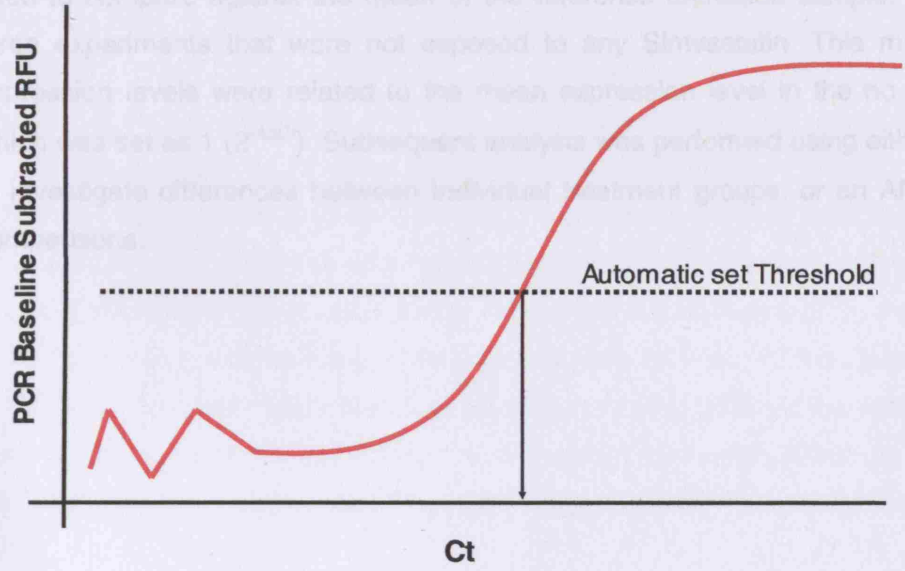
an Eppendorf multichannel pipette. Cells were incubated in 5% CO_2 at 37°C for 48 hours before mRNA extraction.

6.2.3 Two step RT-PCR

Messenger RNA was extracted and cDNA synthesised from each pooled column of wells (described in section 2.6.1). Quantification of *PLA2G7* gene expression (two step RT-PCR) was performed using Taqman and all gene expression assays and conditions are described in section 2.6. The thresholds for each experiment were automatically set by the Taqman SDS software (Fig. 6.2), and checked to make sure that the threshold appeared in the linear (exponential) phase of the graphs of all samples tested (The Ct value was defined as the fractional PCR cycle number at which the fluorescence emitted from a particular well rose above the threshold). Each cDNA sample was run on the Taqman in triplicate, and each individuals RT-PCR experiment was replicated twice on different days from the same cDNA stock to ensure consistent results. Before proceeding with the experiment, one healthy individual's macrophages were cultured and exposed to 0, 10, and 25μM concentrations of simvastatic acid using the pre-defined experimental conditions (each concentration was replicated in eight individual wells of a 96-well plate). Messenger RNA was extracted and cDNA synthesised. The relative expression of three standard reference genes; Glyceraldehydephosphate dehydrogenase (GAPDH), β-actin, and Ubiquitin-C (UBC) were compared in duplicate in these samples using the REST algorithm in the GeNorm v3.4 software (Vandesompele et al., 2002).

GeNorm v3.4 determines the most stable reference (housekeeping) gene from a set of tested genes in a given cDNA sample panel. From this it calculates the gene expression normalisation factor for each cell line based on the geometric mean of a defined set of reference genes. Essentially, for each reference gene, a gene expression stability measure M is defined as the pairwise variation for that gene against all the other genes tested. Previous analysis has suggested that an M value of <1.5 indicates a stably expressed housekeeping gene (Vandesompele et al., 2002).

Fig. 6.2: Automatic threshold set for each set of samples tested. The Ct value entered into the EXCEL database is the point at which the threshold dissects the exponential amplification phase of the sample



6.2.4 Statistical analysis

All data was exported from the SDS Relative quantification software and initially entered onto an EXCEL spreadsheet. Before statistical analysis, all data was normalised to the pre-selected reference gene, and subsequently compared against an un-treated reference group (those samples not exposed to statin).The difference for each sample relative to the reference gene was calculated (following the manufacturers instructions) as follows:-

$$\Delta Ct = (Ct\ PLA2G7) - (Ct\ \text{reference gene})$$

A comparative Ct ($\Delta\Delta Ct$) was obtained for the treated sample relative to the untreated control sample using the formula:-

$$\Delta\Delta Ct = (\Delta Ct\ \text{Treated}) - (\Delta Ct\ \text{Untreated control sample})$$

The amount of mRNA, normalized to an untreated control sample and relative to a calibrator reference gene (assuming 100% efficiency) is given by:-

$$2^{-\Delta\Delta Ct} \text{ or } 2^{-(\Delta Ct\ \text{Stimulated})-(\Delta Ct\ \text{Reference})}$$

All statistical analysis was carried out by myself using SPSS v12.0.1 (SPSS Inc., Chicago USA).The mean of the $\Delta\Delta$Ct for the three replicated experiments in each individual were used to compare against the mean of the reference untreated sample, in this case, the three experiments that were not exposed to any Simvastatin. This meant that all the expression levels were related to the mean expression level in the no-treatment group, which was set as 1 ($2^{-\Delta\Delta Ct}$). Subsequent analysis was performed using either paired T-tests to investigate differences between individual treatment groups, or an ANOVA for overall comparisons.

6.3 Results

6.3.1 Selection of reference gene

When GAPDH, β-actin, and UBC were compared to each other, the GeNorm M value was lowest for UBC (1.09) with GAPDH (1.93) and β-actin (2.53) showing much higher values. Figure 6.3 shows graphically (the SDS amplification plot) the discrepancies between the three housekeeping genes when tested in one individual's macrophages exposed to 0μM, 10μM and 25μM simvastatic acid: the UBC experiment shows a tight clustering of Ct lines at all concentrations (lighter coloured lines reflect higher statin concentrations), suggesting a stably expressed gene under these experimental conditions. Both the GAPDH and β-actin Ct values are spread out, potentially reducing accuracy of the assay. All subsequent RT-PCR experiments were therefore carried out using UBC as the reference gene. The mean intra-assay (within individual) CV of the UBC reference gene in all experiments was 3%, while the mean inter-assay (between different individuals tested) CV was 11%.

Fig 6.3: SDS graphical output of three endogenous 'house-keeping' genes being tested. Colour of lines represent concentration of Simvastatic acid exposure (blue= low, green= high). For each concentration of statin, 8 individual wells were used and duplicated in the subsequent RT-PCR analysis

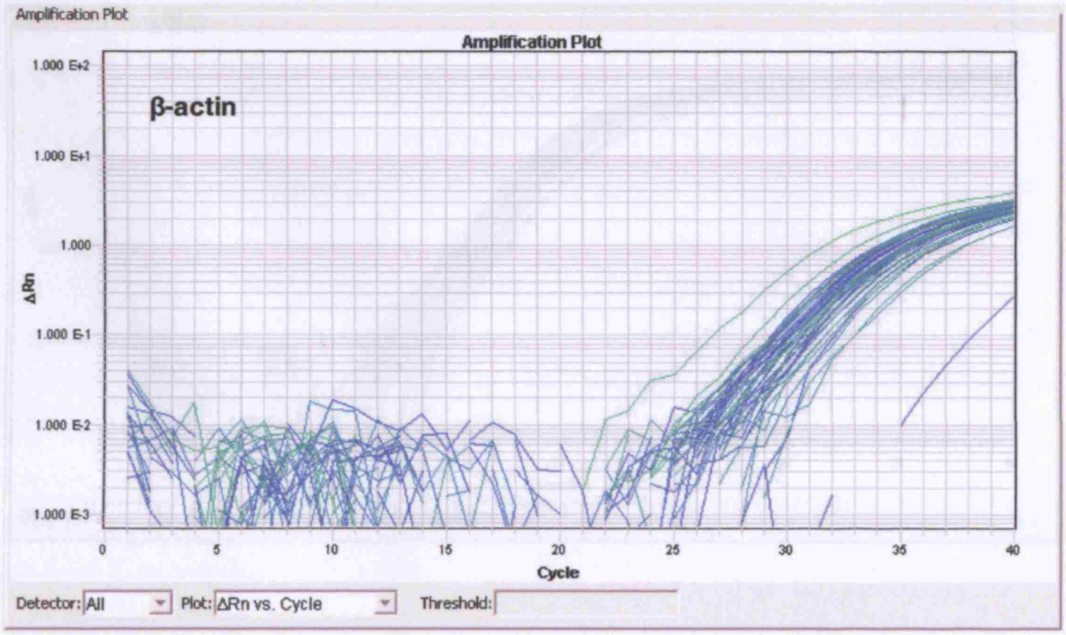
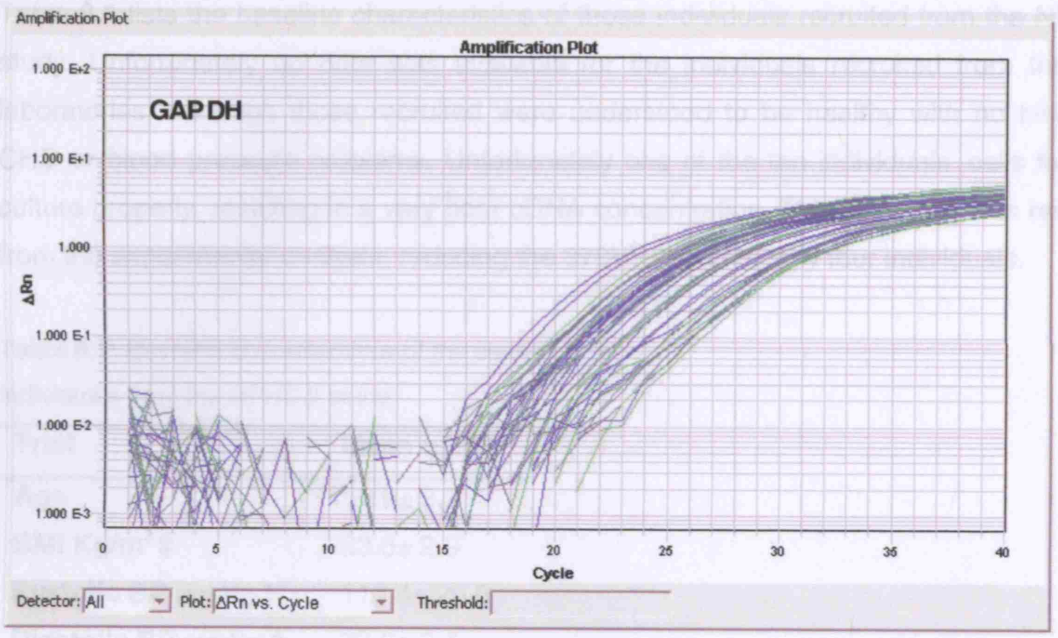
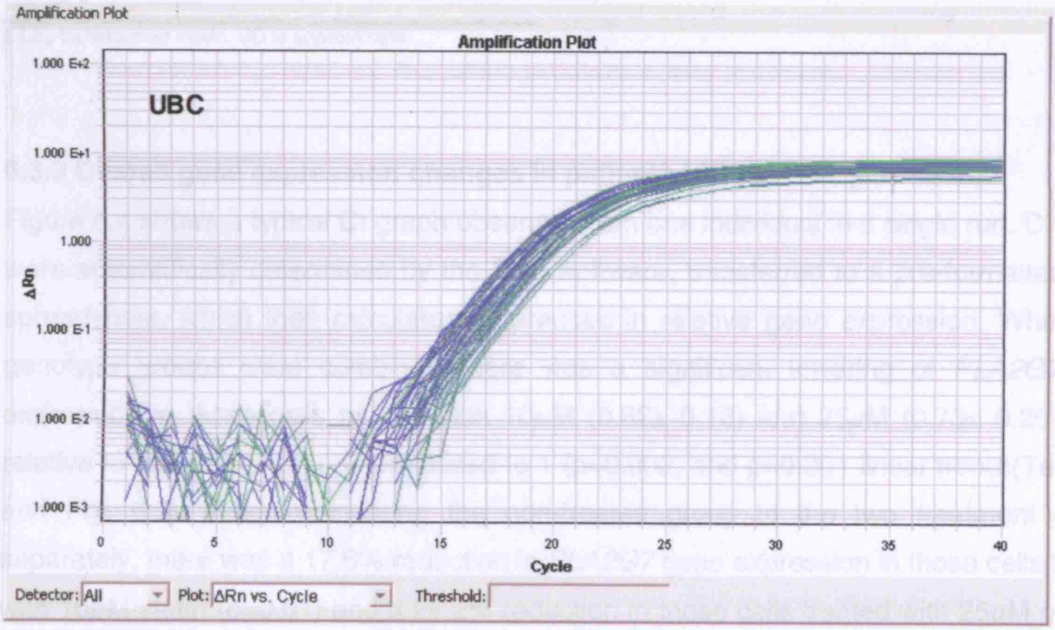


Fig 6.3: Continued





6.3.2 Baseline characteristics of the sample

Table 6.1 lists the baseline characteristics of those individuals recruited from the NPHS II study. Unfortunately no data was available for the individuals recruited from the BHF laboratories, although those recruited were understood to be healthy with no history of CHD or blood pressure problems. Unfortunately one of the ten individuals' cells failed to culture properly, resulting in a very poor cDNA concentration. This individual was removed from the experimental analysis, reducing the 379VV group to only four individuals.

Table 6.1: Baseline characteristics of the seven individuals from the NPHS II study.

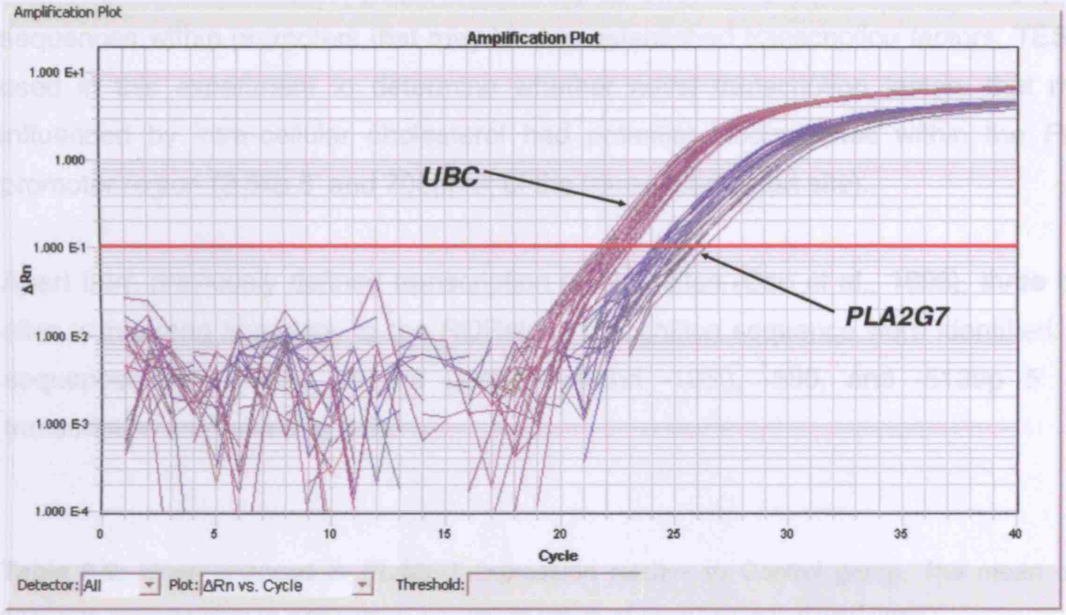
Trait	Mean ±1SD
Age	50.0± 2.3
BMI Kg/m^2 ‡	23.6± 2.9
Systolic BP mmHg ‡	118.4± 20.6
Diastolic BP mmHg ‡	76.9± 9.4
Fibrinogen mg/dL ‡	226.4± 43.3
Cholesterol mmol/L	5.3± 0.9
Triglyceride mmol/L ‡	1.8± 0.7

‡ Log transformed mean. SD is approximate

6.3.3 Overall gene expression changes in patients tested

Figure 6.4 shows a typical Ct graph observed from one individual in a single run. Ct values were automatically determined by the SDS software, transferred to a pre-formatted excel spreadsheet, which then calculated differences in relative gene expression. When both genotype groups were combined there was a significant lowering of *PLA2G7* gene expression in those cells treated with 10μM (0.82± 0.16) and 25μM (0.73± 0.20) statin relative to the control group normalised to 1 (p=0.002, and p=0.001 linear trend)(Table 6.2 and Fig. 6.5). When comparing the non-treated group to the two treatment groups separately, there was a 17.6% reduction in *PLA2G7* gene expression in those cells treated with 10μM statin (p=0.01) and a 27.2% reduction in those cells treated with 25μM of statin (p=0.003) (Table 6.2 and Fig. 6.5).

Fig 6.4: Typical Ct graph obtained from a single patient. The threshold was set automatically by the SDS software.



6.3.4 Results by A379V genotype

Despite the A379V variant being regarded as an activity altering polymorphism, it was important to establish whether the A379V variant influenced the association of *PLA2G7* gene expression changes in response to Simvastatin treatment. Overall there was no heterogeneity of effect between VVs and AAs (p=0.964 for comparison of 10μM treatment, and p=0.167 for 25μM) in terms of reduction in expression. When the AA and VV individuals were compared separately, there was an overall reduction in expression in those AA cells treated with statin (p=0.03, p=0.01 linear trend)(Table 6.2 and Fig. 6.5). Those AA genotype macrophages treated with 10μM statin showed a non-significant 17.2% reduction in *PLA2G7* expression (p=0.16) compared to untreated cells, while cells treated with 25μM statin showed a significantly lower expression (35.2%, p=0.02)(Table 6.2 and Fig. 6.5). Cells homozygous for the 379V allele showed a borderline significant reduction in *PLA2G7* expression when treated with statin (p=0.05, p=0.04 linear trend). Considered separately, VV cells treated with 10μM statin showed a 18.0% lower expression compared to control cells (p=0.01), while those cells treated with 25μM statin showed a non-significant 17.2% lower expression when compared to untreated cells (p=0.13)(Table 6.2 and Fig. 6.5).

6.3.5 TESS transcription binding sites

The TESS search engine (http://www.cbil.upenn.edu/tess/) is able to identify potential sequences within promoters that may bind to established transcription factors. TESS was used in this experiment to determine whether novel transcription factors that may be influenced by intra-cellular cholesterol had potential binding sites within the *PLA2G7* promoter region (3.5kb 5' and 700bp 3' of the transcription start site).

Apart from previously defined transcription binding sites (Cao et al., 1998), three binding sites with strong homology to the RORalpha recognition sequence were identified. These sequences were found on the positive strand -1250, -880, and -513bp 5' of the transcription start site (Fig 6.6).

Table 6.2: Mean changes in *PLA2G7* expression relative to Control group. The mean of three seperate experiments in each statin treatment group of each individual was used.

Genotype (n)	control	10μM (1SD)	25μM (1SD)	p value ANOVA	p value linear trend	p value control vs 10μM T-test	p value control vs 25μM T-test
Overall (9)	1 (0.23)	0.824 (0.16)	0.728 (0.20)	0.002	0.001	0.01	0.003
AA (5)	1 (0.16)	0.828 (0.22)	0.648 (0.20)	0.03	0.01	0.16	0.02
VV (4)	1 (0.26)	0.820 (0.06)	0.828 (0.16)	0.05	0.04	0.01	0.13

Fig 6.5: Relative gene expression of cells exposed to different concentrations of Simvastatic acid relative to controls set at 1 (±1SD). Standard deviation for the control is a mean intra-individual SD of the Ct values before normalisation to the control.

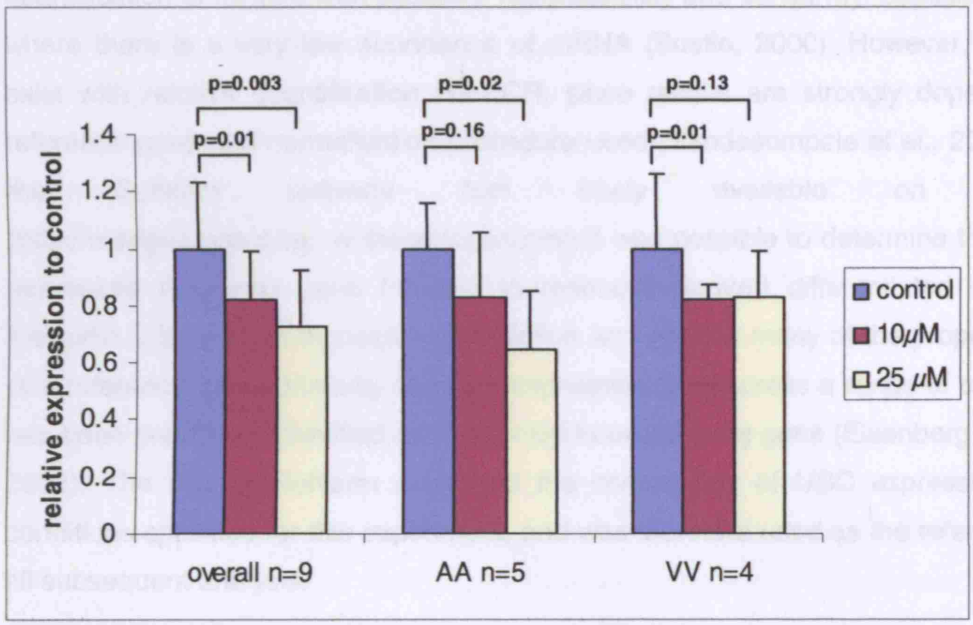
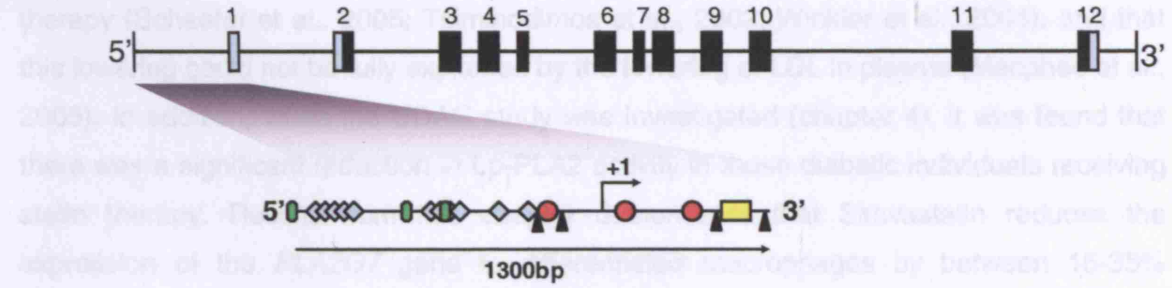


Fig 6.6: Proximal promoter of the *PLA2G7* gene. Green circles represent the potential RORalpha transcription binding factor sequences.



- Myeloid specific factor 2 (MS2)
- Sp 1
- STAT consensus sequence
- Pu. 1 box
- Potential RORalpha binding site

6.4 Discussion

Reverse transcription followed by RT-PCR is a powerful tool for the detection and quantification of mRNA, with excellent reproducibility and sensitivity, especially in samples where there is a very low abundance of mRNA (Bustin, 2000). However, problems still exist with relative quantification RT-PCR, since results are strongly dependent on the reference gene and normalisation procedure used (Vandesompele et al., 2002). By using the GeNorm software tool freely available on the web (http://medgen.ugent.be/~jvdesomp/genorm/) it was possible to determine the most stably expressed reference gene for use in monocyte-derived differentiated macrophages. Ubiquitin C is involved in protein degradation and exhibits many of the properties required of a reference gene, primarily constant expression level across a range of conditions, and has been previously identified as a potential housekeeping gene (Eisenberg and Levanon, 2003). The use of GeNorm confirmed the consistency of UBC expression under the conditions specified for this experiment, and was therefore used as the reference gene for all subsequent analysis.

Simvastatin is regarded as a consistent cholesterol and CHD-risk lowering drug (The Scandinavian Simvastatin Survival Study group, 1994; Heart protection Study Collaborative group, 2002; Pyorala et al., 1997). While previous epidemiological data has shown that lower Lp-PLA2 mass and activity is associated with a variety of statin drug therapy (Schaefer et al., 2005; Tsimihodimos et al., 2002; Winkler et al., 2004), and that this lowering could not be fully explained by the lowering of LDL in plasma (Macphee et al., 2005). In addition, when the UDAC study was investigated (chapter 4), it was found that there was a significant reduction in Lp-PLA2 activity in those diabetic individuals receiving statin therapy. Results from this chapter demonstrate that Simvastatin reduces the expression of the *PLA2G7* gene in differentiated macrophages by between 15-35% depending upon the concentration of statin present in the culture medium. There also appeared to be a strong linear relationship between the concentration of statin applied to the growth media, and *PLA2G7* gene expression. These results are in direct contrast to those of Tsimihodimos et al.(Tsimihodimos et al., 2002), who found that Lp-PLA2 activity was elevated *in vitro* upon Atorvastatin treatment, while mRNA levels stayed constant. However, this group used β-actin as a reference gene, which we found to be a poor (and erratic) reference gene in monocyte derived macrophages.

Individual analysis demonstrated that the A379V variant is having no effect on the association of reduced *PLA2G7* gene expression with Simvastatin treatment. This result was in some ways expected, since published data suggests that the A379V variant's role is related to Lp-PLA2 activity (Kruse et al., 2000; Ninio et al., 2004). However, there was the possibility that the A379V variant could be in LD with other polymorphisms present either in the 3' UTR or promoter region, and that these SNPs could be functional with regards to *PLA2G7* gene expression. Indeed, the T-403C and C-209G SNPs present in the *PLA2G7* promoter have been reported to be in strong LD with the A379V variant (Ninio et al., 2004), although it is unknown whether these SNPs have a functional effect on *PLA2G7* promoter function.

Approximately 70-80% of the plasma enzymatic activity of Lp-PLA2 is associated with LDL particles, with the remainder residing on HDL (Guerra et al., 1997; Stafforini et al., 1987; Tjoelker and Stafforini, 2000). Therefore a reduction in LDL cholesterol levels associated with statin treatment could explain any reduced plasma Lp-PLA2 activity or mass. The results presented here open up the possibility that Lp-PLA2 activity and mass are not just regulated by the LDL-mediated effects of statins, but also by a distinct effect on the transcription of the *PLA2G7* gene in cells of a haemopoietic origin. Statins are known to have many diverse anti-inflammatory actions (Davignon, 2004; Schonbeck and Libby, 2004). *In vitro*, *in vivo* and epidemiological data has shown that statins influence the expression of endothelial adhesion molecules such as Vascular Cell Adhesion Molecule (VCAM) and Inter-Cellular Adhesion Molecule (ICAM) and mediators involved in the thrombotic pathways such as tissue factor, fibrinogen and Plasminogen Activator Inhibitor (PAI) -1 [reviewed in (Schonbeck and Libby, 2004)]. Inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, and IFN-γ are known to be down regulated by statin therapy (Schwartz and Olsson, 2005; Davignon, 2004; Schonbeck and Libby, 2004; Rezaie-Majd et al., 2002), and these cytokines are themselves responsible for the transcriptional regulation of a number of inflammatory genes. The reduction in inflammation observed with statin treatment is illustrated with CRP, where statins are found to be associated with a significant and consistent lowering of CRP levels in human subjects (Ridker et al., 2005; Ridker et al., 1999; Ridker et al., 2001). Other inflammatory molecules such as Cyclooxygenase 2, Serum amyloid A protein, and PPARγ are also thought to be affected by statin therapy (Schonbeck and Libby, 2004).

With regards to the effects on *PLA2G7* expression, Cao et al. identified several STAT consensus sequences within the proximal *PLA2G7* promoter which are thought to be important in IFN-γ mediated effects on expression (Cao et al., 1998)(Fig. 6.6). A reduction in IFN-γ [often seen with statin treatment (Chung et al., 2002; Rosenson et al., 1999)] could therefore explain the lowering of *PLA2G7* expression in those cells treated with Simvastatin. IFN-γ is part of a group of cytokines responsible for mediating the TH1 immune response, a primary inflammatory pathway present in human and mouse atheroma (Frostegard et al., 1999).

Despite the identification of several STAT sequences, the *PLA2G7* promoter may contain other potential transcription binding factors that could influence expression under Statin treatment. The transcription element search system (TESS, http://www.cbil.upenn.edu/tess/) also identified three sites that showed high homology with RORalpha transcription factor binding domains (Fig 6.6). The retinoic acid-related orphan receptor α (RORalpha) is a member of the nuclear hormone receptor family (Willson, 2002). The structure of RORalpha has been elucidated using x-ray crystallography, with a cholesterol ligand binding pocket identified (Kallen et al., 2002). This binding domain is thought to modulate RORalpha transcriptional activity according to intra-cellular concentrations of cholesterol (Kallen et al., 2002; Willson, 2002), and could therefore be affected by the inhibition of HMG CoA-reductase by statins. Mice deficient in RORalpha activity show cerebellar degeneration, but more interestingly they exhibit several vascular phenotypes, in particular, dysfunction of smooth muscle cells and an overall enhanced susceptibility to atherosclerosis (Boukhtouche et al., 2004). There is also evidence that RORalpha is involved in plasma cholesterol metabolism, with evidence that it regulates ApoA-I and ApoC-III transcription (Vu-Dac et al., 1997; Raspe et al., 2001). Lovastatin has been found to be a strong transcriptional inhibitor of RORalpha, since HMG CoA-reductase is an important pathway in the generation of intra-cellular cholesterol, which itself binds and adds stability to the RORalpha protein (Kallen et al., 2002). It is therefore conceivable that *PLA2G7* expression could be suppressed by this pathway when statin is applied. However, as yet undetermined transcription factors responding to statins, with binding sites present in the *PLA2G7* promoter, could be having an effect on mRNA transcription. Further work is clearly needed to determine if the RORalpha binding sites identified by TESS are active *in vitro* and *in vivo*.

In conclusion, the inhibitory effect of Simvastatin on *PLA2G7* gene expression has been clearly demonstrated in monocyte-derived differentiated macrophages. However, there were several limitations to this study. No Lp-PLA2 activity/mass measures were taken from the media, which may have confirmed that a reduction in *PLA2G7* gene expression led to reduced secretion of Lp-PLA2. This experiment while adding a valuable insight into the many pleiotropic effects of statins, does not present a conclusive mechanism by which *PLA2G7* expression is suppressed, and does not replicate accurate physiological conditions. The statin concentration and incubation time selected for this experiment reflects previous *in vitro* work carried out using Simvastatin in Bart Stael's laboratory by Elisabeth Teissier, and also published data from a variety of cell culture studies (Loike et al., 2004; Lee et al., 2004; Rezaie-Majd et al., 2002). However, these conditions could not replicate the *in vivo* physiological exposure that macrophages experience. Despite these limitations, the results of this chapter and those from the UDAC study (Chapter 4) show that statin therapy may represent an important therapeutic element in reducing Lp-PLA2 activity and mass, distinct from the new potent Lp-PLA2 inhibitors currently under development (Blackie et al., 2002; Blackie et al., 2003).

6.5 Summary of Results

i) Simvastatin treatment of differentiated macrophages in culture resulted in a 15-35% reduction in *PLA2G7* gene expression compared to untreated cells.

ii) There appeared to be no heterogeneity of effect of A379V genotype on the effect of Simvastatin on *PLA2G7* gene expression

iii) TESS identified three homologous binding sites to the RORalpha transcription factor, which itself is modulated by intra-cellular cholesterol, and plays an important regulatory role in plasma cholesterol pathways and inflammatory responses.

CHAPTER 7

THE DETECTION OF COMMON HAPLOTYPES IN THE SECRETORY PHOSPHOLIPASE A2 GENES *PLA2G2A* AND *PLA2G5* AND THEIR ASSOCIATION WITH ATHEROSCLEROSIS

7.1 Introduction

To date more than 15 different Phospholipase A2 enzyme family members have been identified that exhibit a wide range of physiological functions (Jaross et al., 2002; Six and Dennis, 2000). As such, investigating all of the PLA2 superfamily enzymes with regards to their role in the progression of atherosclerosis would be very challenging. This thesis has tried to specifically assess the contribution of three of these PLA2 enzymes to atherosclerosis. All three PLA2 enzymes are closely linked through their specific activities regarding the oxidative modification of lipoproteins and inflammation; processes that are important in the generation of an atherosclerotic plaque (Ross, 1999).

Previous chapters in this thesis have investigated whether the Lipoprotein-associated PLA2 gene *PLA2G7* directly influences individual differences in markers of atherosclerosis, plasma measures of Lp-PLA2 activity, and risk of CHD. Chapter 7 of this thesis is dedicated to determining the contribution to atherosclerosis of two closely associated secretory PLA2 enzymes, sPLA2 IIA and V. By using novel genetic approaches distinct from direct candidate SNP analysis (as used in the analysis of the *PLA2G7* gene), the role of sPLA2 with regards to atherosclerosis could be investigated, and a useful comparison made of these two genetic approaches in investigating candidate gene associations.

7.1.1 Secretory PLA2 enzymes role in atherosclerosis

Many of the properties of the two sPLA2 enzymes under investigation in this thesis are shared, although subtle differences do exist. Chapter 1 (section 1.3.4) gives a comprehensive description of the properties of both the sPLA2 IIA and V enzymes with regards to their transcription, expression, substrate preferences and potential involvement in atherosclerosis. However, when considering sPLA2 function in the progression of atherosclerosis, much of the published work to date has concentrated on the role of the IIA enzyme. Both sPLA2 enzymes represent exciting targets for investigation, since they exhibit pro-atherogenic properties both in the circulation and the arterial wall (Hurt-Camejo et al., 2001).

As with all PLA2 family members, the sPLA2 enzymes under investigation in this thesis are able to hydrolyse the sn-2 ester bond of phospholipids, generating significant

quantities of lysophospholipids and non-esterified free fatty acids (NEFA). However, differences exist regarding the ability of all three PLA2 enzymes under investigation to hydrolyse certain phospholipids species (see section 1.4.3.3) (Han et al., 1998; Jaross et al., 2002; Kim et al., 2000). Apart from the production of two powerful mediators of inflammation (Hurt-Camejo and Camejo, 1997; Oestvang et al., 2004), the actions of sPLA2 enzymes are an important feature in the modification of lipoproteins. Within the circulation, hydrolysis of phospholipids present on the surface of LDL by sPLA2 leads to the generation of smaller, denser LDL particles that are more susceptible to oxidation (Sartipy et al., 1999). The presence of sphingomyelin (SPH) and proteoglycans are important in regulating sPLA2 activity, since SPH confers stability to phospholipid bi-layers, preventing effective hydrolysis by sPLA2 enzymes (Subbaiah et al., 1999). SPH also competitively binds to the active site of sPLA2, reducing its capacity to hydrolyse other phospholipids species (Koumanov et al., 1997).

The activity of sPLA2 in the arterial intima suggests that these enzymes may mediate strong pro-inflammatory and pro-atherogenic effects. There is convincing evidence that sPLA2 localises to atherosclerotic plaques (Menschikowski et al., 1995a). A strong sPLA2 IIA immunoreactivity has been observed in the arterial media in atherosclerotic vessels (Hurt-Camejo et al., 1997). Diseased arteries showed localisation of the enzyme to SMC, macrophages, extra-cellular matrix areas and to regions close to the necrotic core of the plaque (Romano et al., 1998). Within the arterial wall, two potential mechanisms exist by which sPLA2 mediates a pro-atherogenic effect: Firstly, it has been shown *in vitro* that sPLA2 is able to cause the aggregation but not fusion of LDL particles (Hakala et al., 1999)(LDL particles fused when sPLA2 and sphingomyelinase (SMase) were added together (Oorni et al., 1998)), and leads to a marked susceptibility to lipid peroxidation (Sartipy et al., 1999), both key steps with regards to macrophage growth (foam cell formation) and the generation of an atherosclerotic plaque. Proteoglycans are able to interact with sPLA2 via their glycosaminoglycan moiety, and there is evidence that this enhances the hydrolysis of LDL phospholipids and leads to retention of LDL within the arterial intima (Sartipy et al., 1996). Table 1.6 gives a comprehensive summary of the observed effects of sPLA2 on LDL particles.

The second major impact of sPLA2 activity in the arterial wall is the production of pro-inflammatory mediators. Most of the products produced from the action of sPLA2 are

successfully transferred to albumin. However, *in vitro* analysis has shown that over half the products stay with the lipoprotein particles (Gorshkova et al., 1996). Hydrolysis of phospholipids leads to the release of high local concentrations of non-esterified free fatty acids (NEFA), oxidised NEFAs, and Lysophospholipids, which may affect the function of a range of vascular cells at the sites of LDL accumulation. These products may either act as intracellular second messengers or can be further metabolised into pro-inflammatory lipid mediators like eicosanoids (Balboa et al., 1996), PAF and lysophosphatidic acid (Snitko et al., 1997). Lyso-PC itself is chemo attractant for monocytes and T-lymphocytes (Asaoka et al., 1993), induces the expression of growth factors and adhesion molecules in endothelial cells, is mitogenic for macrophages and vascular smooth muscle cells (Pruzanski et al., 2001), and inhibits endothelium-dependent relaxation and endothelial-cell motility. Lyso-PC also alters sub-endothelial heparin sulphate proteoglycan making it more adhesive to monocytes. Beside these effects, there is evidence that the extra cellular generation of lyso-PC by secretory PLA2 may promote tissue inflammation and haemostatic disturbances (Hurt-Camejo and Camejo, 1997).

Figure 1.16 gives an overview of the characterised effects of sPLA2 enzymes on the progression of atherosclerosis. As already stated, both sPLA2 enzymes represent potent pro-inflammatory enzymes that could be centrally involved in atherosclerosis. Transgenic mouse models have further confirmed this with *PLA2G2A* mice showing a dramatic increase in atherosclerosis compared to wild-type littermates (Ivandic et al., 1999; Leitinger et al., 1999). Previous epidemiological data has also shown that sPLA2 IIA is an acute phase reactant with circulating levels significantly elevated in those patients with CHD (Hurt-Camejo et al., 2001; Kugiyama et al., 1999; Liu et al., 2003). In particular, recently published data from the prospective EPIC-norfolk study suggested that elevated levels of sPLA2 IIA are associated with an increased risk of CHD in apparently healthy men and women (Boekholdt et al., 2005). However, despite robust associations of sPLA2 with CHD and plausible biological mechanisms to support this link, observational studies to date could be affected by confounding or reverse-causation in a similar way to those studies investigating the relationship of Lp-PLA2 and CHD. In an effort to overcome this, the focus of my PhD thesis was to examine the relationship between sPLA2 levels (IIA only) and variation in the *PLA2G2A* and *PLA2G5* genes, as well as markers of atherosclerosis that may be influenced by the actions of these enzymes.

7.1.2 The concept of indirect candidate associations and Haplotype analysis

Direct candidate SNP approaches suffer from several inherent difficulties. Single variants with low allele frequencies are difficult to study since the sample size required to detect a moderate effect in a rare variant can be prohibitively large (Suh and Vijg, 2005). Another problem is determining the precise functionality of the variant under investigation. This has been particularly evident in analysis of the A379V variant in the *PLA2G7* gene in this thesis and other published association studies (Kruse et al., 2000; Ninio et al., 2004). In addition, single SNPs under investigation found to be associated with CHD may be part of a larger set of several susceptibility alleles (Pritchard and Cox, 2002). If a complex disease such as atherosclerosis is influenced by multiple rare variants in a gene, each of minor or modest effect, each risk-increasing variant may be too rare to achieve a level of significance (Suh and Vijg, 2005). These factors make investigating associations with a single SNP problematic. In particular, positive results that stand the test of time by being consistently detected by other researchers have proven to be difficult to achieve (Farrall and Morris, 2005; Ioannidis et al., 2001).

Recently, characterisation of patterns of LD across the human genome has become a highly active area of research, culminating in the generation of a haplotype map [HapMap (The International HapMap Consortium, 2003)]. Examination of a high density map of markers has revealed an elegantly simple pattern to human genetic variation: blocks of variable length over which only a few common haplotypes are observed, punctuated by sites at which recombination could be inferred in the history of the sample (Gabriel et al., 2002). This is particularly evident in one segment of the major histocompatibility complex (MHC) on chromosome 6, where 'hot-spots' of meiotic recombination coincided with boundaries between such blocks (Jeffreys et al., 2001). The significant levels of LD found within the haplotype blocks present a useful new tool in genotyping studies, avoiding the need to genotype every single SNP present within a specific region when examining associations (Suh and Vijg, 2005).

With the emergence of consensus sequence information and databases containing gene SNP and haplotype information, it has become feasible to consider haplotype analysis of whole gene regions and sometimes clusters of genes involved in particular pathways. This 'indirect' approach is where a set of sequence variants in the genome act as genetic markers to detect association between a particular genomic region and disease, whether

or not the markers themselves have functional effects. The search for the causative variants could then be limited to the regions showing association with the disease (The International HapMap Consortium, 2003). The indirect approach is possible because a few selected SNPs are able to capture most of the human sequence variation. Haplotypes are the particular combinations of alleles observed in a population. When a new mutation arises, it does so on a specific chromosomal haplotype. The association between each mutant allele and its ancestral haplotype is disrupted only by mutation and recombination in subsequent generations (Gabriel et al., 2002). Thus it should be possible to track each variant allele in the population by identifying, through the use of a few selected variants, the particular ancestral segment on which it arose (Gabriel et al., 2002). The use of a small set of variants that capture most of the common patterns of variation in the genome enables any gene to be tested for association with a particular disease with a high likelihood that such an association will be detectable if it exists (The International HapMap Consortium, 2003).

Figure 7.1 gives a demonstration by which selected variants, termed 'tagging SNPs', can be selected and used in association studies. In the region of 90% of sequence variation among individuals is due to common variants (Kruglyak, 1999)(Fig 7.1 A). Every variable site results from a single historical mutation event and each new allele is initially associated with the other alleles that happened to be present on the particular chromosomal background on which it arose; this is termed a haplotype (Fig. 7.1 B). New haplotypes can be formed by additional mutations or by recombination (Paabo, 2003). The selection of tagging SNPs (tSNPs) is possible because of the strong allelic association (LD) that is seen in a particular region. By carefully choosing SNPs it is possible to identify each of the common haplotypes present in a region (Johnson et al., 2001; Zhang et al., 2002)(Fig. 7.1 C).

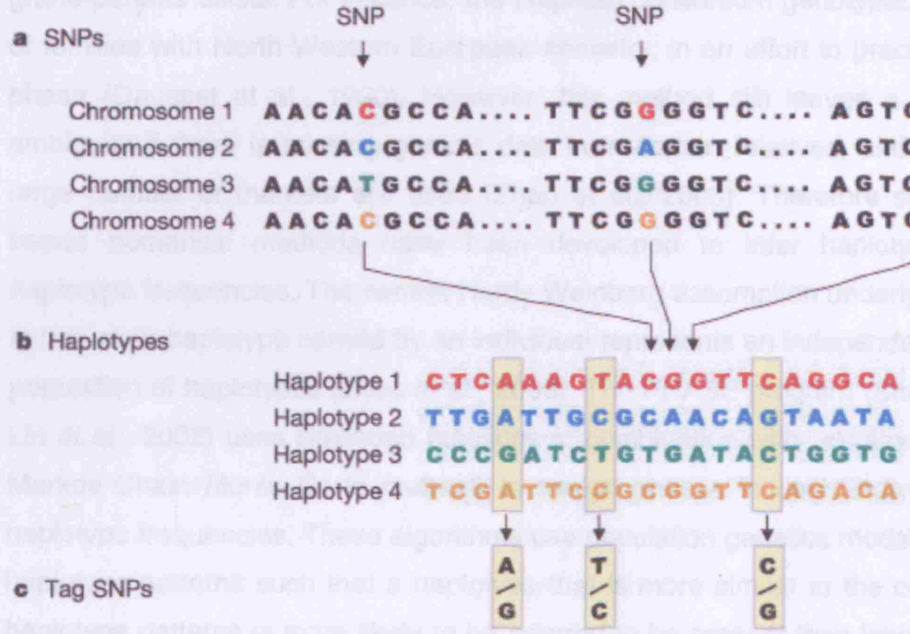
Haplotype analysis offering a statistically powerful approach to gene association studies (Goldstein et al., 2003; Goldstein, 2001) involves the following essential steps: an appropriate selection of candidate genes in pathways where evidence already exists of its impact on the disease of interest (for example mouse model data); determination of the haplotype structure of this gene in control populations; the selection of tSNPs in an attempt to represent the common haplotypes in each gene; testing for association between

phenotypes and haplotype status; and scrutinising positively associated haplotypes in order to determine the truly functional variant.

However, despite the exciting prospects of tSNP approaches, there are still some concerns over its accuracy at detecting all of the common sequence variation within a specified region. The use of tSNPs is reliant on there being relatively strong LD across the region under investigation. Several studies to date have identified that patterns of LD are not always consistent (Dunning et al., 2000), and that this is particularly true when considering different ethnic and geographical population groups (Suh and Vijg, 2005). There is also a question relating to whether a few genotyped SNPs are enough to fully capture haplotype structures and their potential associations with disease, although analysis of the power lost by using a tSNP approach has been found to be relatively small (~4%)(Zhang et al., 2002). But more fundamentally, there is an assumption made that a small number of ancient SNPs that occur at high frequencies in all populations are responsible for susceptibilities to complex common diseases such as atherosclerosis i.e., the common disease/common variant hypothesis (CD/CV) (Suh and Vijg, 2005; Zondervan and Cardon, 2004). If complex diseases were found to be due to a large number of rare variants at many loci, the tagging SNP approach would obviously fail, as no single haplotype would be strongly associated with complex traits (Pritchard, 2001), and the contribution of most individual variants would be too small to detect (Pritchard and Cox, 2002). A final problem of haplotype analysis is the ability to find the truly functional variant once a haplotype has been found to be associated with disease (Suh and Vijg, 2005).

The concept of haplotype analysis does however, circumvent the currently un-achievable aim of genotyping every single SNP in a gene of interest in order to guarantee that no important gene variants are avoided (The International HapMap Consortium, 2003). It also presents a powerful mechanism by which to first screen genetic regions of interest for associations with disease. Very few genotype association studies have been carried out in either the *PLA2G2A* or *PLA2G5* genes, with one previous study investigating the association of various *PLA2G2A* polymorphisms with familial adenomatous polyposis and sporadic colorectal tumours (Tomlinson et al., 1996). The lack of previous data concerning the genetics of these two genes meant that using a haplotype tagging approach offered a powerful method by which to further investigate the contribution of these two genes to the progression of atherosclerosis in humans.

Fig 7.1: The theoretical determination of tagging SNPs for use in genetic association studies (diagram courtesy of (The International HapMap Consortium, 2003))



7.1.3 Haplotype phase

A further obstacle to haplotype analysis is that of inferring haplotype 'phase'. An individual's genotype may not uniquely define that individual's haplotype. If one considers two SNPs, each with two possible alleles, the first SNP being either *A* or *a*, the second SNP being *B* or *b*. If the genotype of an individual was found to be *AaBb*, there are two possible sets of haplotypes, corresponding to which pairs happen to occur on the same chromosome:

	Chromosome 1 Haplotype	**Chromosome 2 Haplotype**
Haplotype set 1	*AB*	*ab*
Haplotype set 2	*Ab*	*aB*

As such, more information would be required to determine which particular set of haplotypes occur in the individual (i.e. which alleles appear on the same chromosome). This 'phase' can be achieved by looking at pedigrees where information from parents and grand-parents exists. For instance, the HapMap consortium genotyped the CEPH sample of families with North Western European ancestry, in an effort to precisely define genetic phase (Dausset et al., 1990). However, this method still leaves a significant level of ambiguity if there is missing genetic data from certain relatives, and especially when a large number of markers are used (Zhao et al., 2003). Therefore several assumption-based numerical methods have been developed to infer haplotypes and estimate haplotype frequencies. The central Hardy-Weinberg assumption underlying these methods is that each haplotype carried by an individual represents an independent sample from the population of haplotypes (Zhao et al., 2003). The PHASE program (Stephens et al., 2001; Lin et al., 2002) uses likelihood functions in combination with an algorithm (such as the Markov Chain Monte Carlo method) to assign phases in each individual and estimate haplotype frequencies. These algorithms use population genetics models to relate different haplotype patterns such that a haplotype that is more similar to the commonly observed haplotype patterns is more likely to be inferred to be present than less similar haplotypes (Zhao et al., 2003).

7.1.4 Bioinformatics resources for Haplotype analysis

Previously, haplotype analysis was dependent on several laborious steps that lead to the identification of suitable tagging SNPs. As already described, a first step in using this technique is the determination of a haplotype structure and frequency. This would normally require the genotyping of every single identified variant within a region of interest in a carefully selected control population group, followed by statistical analysis using algorithms such as PHASE. Previous web-based databases such as dbSNP (www.ncbi.nlm.nih.gov/entrez/query.fcgi) and the CHIP 'SNPper' tool (http://snpper.chip.org/) provide extensive data regarding the location and frequency of common variants. However, since both resources are reliant on submitted genotype information from different population groups, there is no way of determining haplotype information. However, several resources have recently been made available on the internet that do have haplotype information and enable rapid selection of tSNPs in certain genes, reducing the need to genotype all the genetic variants present in a gene of interest.

For this thesis, the National Institute of Environmental Health Sciences (NIEHS) Environmental Genome Project (EGP) resource was utilised (http://egp.gs.washington.edu/). The EGP is a collaborative effort that has focused on examining the relationships between environmental exposures, inter-individual sequence variation in human genes and disease risk in human populations (Livingston et al., 2004). The EGP to date has re-sequenced 213 environmental response genes involved in DNA repair, cell cycle regulation, apoptosis, and metabolism for SNPs. Many of these genes were hypothesised to be functional from previous studies associating loss of function mutations with disease (Livingston et al., 2004). On average, 86% of each gene of interest, including a substantial amount of introns, all exons, and 1.3 kb 5' and 3', were scanned for variations in 90 samples of the Polymorphism Discovery Resource (PDR) panel (Livingston et al., 2004). The PDR resource is comprised of cell lines and DNA samples from 450 unrelated individuals, male and female, mainly of European Caucasian decent (Collins et al., 1998). In addition to the complete set, a predefined nested subset of 90 samples encompassing the same range of diversity as the complete set was used in the screening process for the EGP study (Livingston et al., 2004). Since the PDR resource was made up of unrelated individuals, PHASE v2 (Stephens et al., 2001; Lin et al., 2002) was used to infer haplotype frequencies in each gene screened. All data was then made freely available on the web (http://egp.gs.washington.edu/).

7.1.5 Aims of this chapter

The EGP resource has complete PHASE data relating to both the *PLA2G2A* and the *PLA2G5* gene region. Suitable tSNPs were selected using a statistical regression tool (STRAM) that selects the minimum number of SNPs required to infer the allelic state of all the common SNPs in the gene of interest (Stram, 2005). Using this tagging SNP approach, I then examined the relationship between serum sPLA2 (IIA measures only) levels, inferred common haplotypes in both genes, and intermediate phenotypes in T2DM individuals in the UDAC study. By using these methods I intended to begin establishing the relationship of both these genes with atherosclerosis.

7.2 Methods

7.2.1 The generation of haplotypes using the NIEHS EGP resource

The EGP website was used to ascertain the typical haplotype frequencies in both sPLA2 genes under investigation (Fig. 7.2). The websites for the *PLA2G2A* and *PLA2G5* genes gives a full appraisal of all genotyping and haplotyping data, including PHASE v2 output files. All genotyping data for both genes relates to that of the PDR 90 population.

Fig. 7.2: Screen shots taken from the NIEHS EGP website for the *PLA2G2A* (http://egp.gs.washington.edu/data/*PLA2G2A*/) and *PLA2G5* genes (http://egp.gs.washington.edu/data/*PLA2G5*/). Circled in each shot is the PHASE v2 output link used to determine inferred haplotype frequencies.

7.2.2 STRAM selection of tagging SNPS in both *PLA2G2A* and *PLA2G5* genes

Figures 7.3 and 7.4 show the PHASE v2 output obtained from the EGP website for both *PLA2G2A* and *PLA2G5* genes. Each row represents a different inferred haplotype based on the SNPs genotyped in the PDR 90 population. Inferred haplotype frequency is also given. The PHASE v2 data was converted into binary 1 or 0 depending on the genotype and entered into the STRAM program which uses a pairwise r^2 method to determine the least number of SNPs needed to infer the complete allelic state of the SNPs entered into the program (Stram, 2005). A cut off for the detectable haplotype variance was selected at ≥95%. All the SNPs shown in Figures 7.3 and 7.4 were entered into STRAM, and those variants selected as potential tSNPs by the STRAM software are highlighted in red.

Fig 7.3: PHASE v2 output obtained from the EGP analysis of SNPs in the *PLA2G2A* gene region. Genotypes are shown as they appear 5' to 3' of the *PLA2G2A* gene. Each row represents a different inferred haplotype combination (Haplotype frequency is shown on the right hand side). Those SNPs selected by STRAM as suitable tSNPs are highlighted in red.

```
************************************************************
****                                                    ****
****           Output from PHASE v2.0                   ****
****  Code by M Stephens, with contributions from N Li  ****
****                                                    ****
************************************************************

BEGIN INPUT_SUMMARY
Number of Individuals: 90
Number of Loci: 17
Positions of loci: 1309 1603 2594 2727 2986 3808 3911 3947 4016 4088 5266 5722
6909 6946 7092 7838 8329
END INPUT_SUMMARY

List of haplotypes found in best reconstruction, with counts.
(See file plg2a.phase.out_freqs for haplotype population frequency estimates)

BEGIN LIST_SUMMARY
         1 TDACTAGCCGTGCATTT    1.000000
         2 TDACTAGCCGTGCATTC   14.000000
         3 TDACTAGCCGTGCTTTT   30.000000
         4 TDACTAGCCGTGCTGTT    2.000000
         5 TDACTAGCCGTGCTGCT    6.000000
         6 TDACTAGCCGTATTGCT    2.000000
         7 TDACTAGCCGGGCTTTT    4.000000
         8 TDACGAGCCGTGCATTT    2.000000
         9 TDACGAGCCGTGCTTTT    4.000000
        10 TDACGAGCCGTGCTTCT    1.000000
        11 TDACGAGCCGTGCTGCT    2.000000
        12 TDACGAGCCGTATTGCT    2.000000
        13 TDACGAGCCGGGCATTT    2.000000
        14 TDACGAGCCGGGCATTC    5.000000
        15 TDACGACCCGTGCTTTT    2.000000
        16 TDACGACCCGTATTGCT    8.000000
        17 TDACGACCCATGCATTC    1.000000
        18 TDACGACCCATGCTGCT    1.000000
        19 TDACGACCCATATTGCT    3.000000
        20 TDACGACCCAGGCATTC    2.000000
        21 TDAGGAGCCGTGCTTTT    1.000000
        22 TDAGGGGTTGTGCTTTT    3.000000
        23 TIACTAGCCGTGCTTTT    2.000000
        24 TIACGAGCCGTGCTTTT    5.000000
        25 TIACGAGCCGTGCTGCT   14.000000
        26 TIACGAGCCGTATTGCT    1.000000
        27 TIACGAGCCGGGCATTC    1.000000
        28 TIACGACCCGTGCTTTT    2.000000
        29 TIACGGGCCGTGCTTTT    1.000000
        30 TIACGGGCCGTGCTGCT    1.000000
        31 TIACGGGTTGTGCTGCT    2.000000
        32 TIAGGGGTTGTGCTTTT    1.000000
        33 TICCGAGCCGTGCTGCT    1.000000
        34 TICCGGGCCGTGCTTTT    2.000000
        35 CDACGAGCCGGGCTGCT    1.000000
        36 CIACTAGCCGTGCATTC    1.000000
        37 CIACTAGCCGTGCTTTT    7.000000
```

```
        38 CIACGAGCCGTGCTGCT    1.000000
        39 CIACGAGCCGTACTTTT    1.000000
        40 CIACGAGCCGTATTGCT    4.000000
        41 CIACGACCCGGGCATTC    1.000000
        42 CIACGACCCATATTGCT    2.000000
        43 CIACGGGCCGGGCATTC    1.000000
        44 CIAGGGGTTGTGCATTT    2.000000
        45 CIAGGGGTTGTGCATTC    1.000000
        46 CIAGGGGTTGTGCTTTT   16.000000
        47 CIAGGGGTTGTGCTGCT    3.000000
        48 CIAGGGGTTGGGCTTTT    1.000000
        49 CICCGGGCCGTGCTTTT    7.000000
END LIST_SUMMARY
```

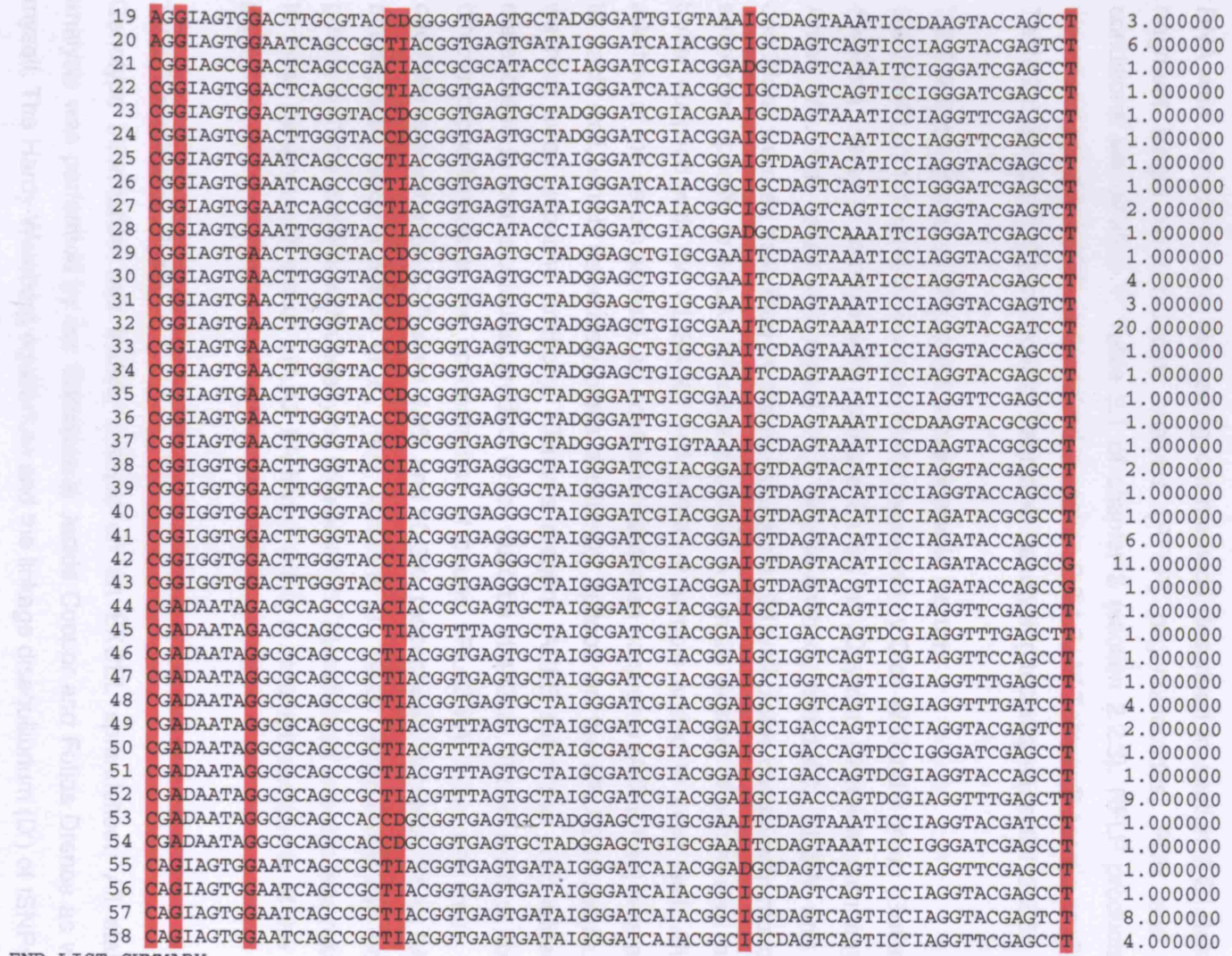
Fig 7.4: PHASE v2 output obtained from the EGP analysis of SNPs in the *PLA2G5* gene region. Genotypes are shown as they appear 5' to 3' of the *PLA2G5* gene. Those SNPs selected by STRAM as suitable tSNPs are highlighted in red.

```
************************************************************
****                                                    ****
****            Output from PHASE v2.0                  ****
****  Code by M Stephens, with contributions from N Li  ****
****                                                    ****
************************************************************

BEGIN INPUT_SUMMARY
Number of Individuals: 90
Number of Loci: 75
Positions of loci: 100 196 286 464 567 591 662 998 1114 1347 1373 1432 1856 1939 2108 2414 2500 2742 3011 3175 3278
3410 3751 3879 5690 6054 6808 7203 7304 7376 7385 7389 7466 7492 7779 7797 8710 8754 8755 8843 9222 9865 10128
10307 10662 11186 11286 11790 12605 12881 13222 13244 13429 13532 13780 14075 14095 15496 15935 16010 16033 18588
18726 18940 19278 19318 19800 20178 21495 21579 22853 22909 23025 23139 24043
END INPUT_SUMMARY

List of haplotypes found in best reconstruction, with counts.
(See file plag5.phase.out_freqs for haplotype population frequency estimates)

BEGIN LIST_SUMMARY
        1 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGACCGIACGGAIGCDAGTCAAAITCIGGGAACGATCCT    1.000000
        2 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGACCGIACGGAIGCDAGTCAAAITCIGGGTACGATCCT    3.000000
        3 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGAIGCDAGCCAGTDCCIAGGTACGATCCT    2.000000
        4 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAAAITCIGGGATCGAGCCT   24.000000
        5 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAAAITCDGGGATCGAGCCT    1.000000
        6 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAATICCIGGGATCGAGCCT    2.000000
        7 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAGTICCIAGGTTCGAGCCT    1.000000
        8 AGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGDACGGAIGCDAGTCAAAITCIGGGATCGAGCCT   11.000000
        9 AGGIAGCGGACTCAGCCGACIACCGCGCATACTCIAGGATCGDACGGAIGCDAGTCAAAITCIGGGATCGAGCCT    1.000000
       10 AGGIAGCGGACTCAGCCGCCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGATCCT    2.000000
       11 AGGIAGTGGACTCAGCCGACIACCGTGAATACCCIAGGATCGIACGGAIGCDAGTCAATICCIGGGATCGAGCCT    1.000000
       12 AGGIAGTGGACTCAGCCGACIACCGTGAATACCCIGGGATCGIACGGAIGCDAGTCAATICCIGGGTTCGAGCCT    1.000000
       13 AGGIAGTGGACTCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTACGAGTCT    1.000000
       14 AGGIAGTGGACTTGGGTACCDGGGGTGAGTGCTADGGGATTGIGTGAAIGCDAGTAAATICCDAAGTACGAGCCT    2.000000
       15 AGGIAGTGGACTTGCGTACCDGGGGTGAGTGCTADGGGATTGIGCGGCIGCDAGTCAGTICCIAGGTACGAGTCT    1.000000
       16 AGGIAGTGGACTTGCGTACCDGGGGTGAGTGCTADGGGATTGIGTGAAIGCDAGTAAATICCDAAGTACGCGCCT   11.000000
       17 AGGIAGTGGACTTGCGTACCDGGGGTGAGTGCTADGGGATTGIGTAAAIGCDAGTAAATICCIAGGTACGAGCCT    1.000000
       18 AGGIAGTGGACTTGCGTACCDGGGGTGAGTGCTADGGGATTGIGTAAAIGCDAGTAAATICCDAAGTACGCGCCT    4.000000
```


19 AGGIAGTGGACTTGCGTACCDGGGGTGAGTGCTADGGGATTGIGTAAAIGCDAGTAAATICCDAAGTACCAGCCT 3.000000
20 AGGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTACGAGTCT 6.000000
21 CGGIAGCGGACTCAGCCGACIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAAAITCIGGGATCGAGCCT 1.000000
22 CGGIAGTGGACTCAGCCGCTIACGGTGAGTGCTAIGGGATCAIACGGCIGCDAGTCAGTICCIGGGATCGAGCCT 1.000000
23 CGGIAGTGGACTTGGGTACCDGCGGTGAGTGCTADGGGATCGIACGAAIGCDAGTAAATICCIAGGTTCGAGCCT 1.000000
24 CGGIAGTGGACTTGGGTACCDGCGGTGAGTGCTADGGGATCGIACGGAIGCDAGTCAATICCIAGGTTCGAGCCT 1.000000
25 CGGIAGTGGAATCAGCCGCTIACGGTGAGTGCTAIGGGATCGIACGGAIGTDAGTACATICCIAGGTACGAGCCT 1.000000
26 CGGIAGTGGAATCAGCCGCTIACGGTGAGTGCTAIGGGATCAIACGGCIGCDAGTCAGTICCIGGGATCGAGCCT 1.000000
27 CGGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTACGAGTCT 2.000000
28 CGGIAGTGGAATTGGGTACCIACCGCGCATACCCIAGGATCGIACGGADGCDAGTCAAAITCIGGGATCGAGCCT 1.000000
29 CGGIAGTGAACTTGGCTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGATCCT 1.000000
30 CGGIAGTGAACTTGGGTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGAGCCT 4.000000
31 CGGIAGTGAACTTGGGTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGAGTCT 3.000000
32 CGGIAGTGAACTTGGGTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGATCCT 20.000000
33 CGGIAGTGAACTTGGGTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACCAGCCT 1.000000
34 CGGIAGTGAACTTGGGTACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAGTICCIAGGTACGAGCCT 1.000000
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41 CGGIGGTGGACTTGGGTACCIACGGTGAGGGCTAIGGGATCGIACGGAIGTDAGTACATICCIAGATACCAGCCT 6.000000
42 CGGIGGTGGACTTGGGTACCIACGGTGAGGGCTAIGGGATCGIACGGAIGTDAGTACATICCIAGATACCAGCCG 11.000000
43 CGGIGGTGGACTTGGGTACCIACGGTGAGGGCTAIGGGATCGIACGGAIGTDAGTACGTICCIAGATACCAGCCG 1.000000
44 CGADAATAGACGCAGCCGACIACCGCGAGTGCTAIGGGATCGIACGGAIGCDAGTCAGTICCIAGGTTCGAGCCT 1.000000
45 CGADAATAGACGCAGCCGCTIACGTTTAGTGCTAIGCGATCGIACGGAIGCIGACCAGTDCGIAGGTTTGAGCTT 1.000000
46 CGADAATAGGCGCAGCCGCTIACGGTGAGTGCTAIGGGATCGIACGGAIGCIGGTCAGTICGIAGGTTCCAGCCT 1.000000
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49 CGADAATAGGCGCAGCCGCTIACGTTTAGTGCTAIGCGATCGIACGGAIGCIGACCAGTICCIAGGTACGAGTCT 2.000000
50 CGADAATAGGCGCAGCCGCTIACGTTTAGTGCTAIGCGATCGIACGGAIGCIGACCAGTDCCIGGGATCGAGCCT 1.000000
51 CGADAATAGGCGCAGCCGCTIACGTTTAGTGCTAIGCGATCGIACGGAIGCIGACCAGTDCGIAGGTACCAGCCT 1.000000
52 CGADAATAGGCGCAGCCGCTIACGTTTAGTGCTAIGCGATCGIACGGAIGCIGACCAGTDCGIAGGTTTGAGCTT 9.000000
53 CGADAATAGGCGCAGCCACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIAGGTACGATCCT 1.000000
54 CGADAATAGGCGCAGCCACCDGCGGTGAGTGCTADGGAGCTGIGCGAAITCDAGTAAATICCIGGGATCGAGCCT 1.000000
55 CAGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGADGCDAGTCAGTICCIAGGTTCGAGCCT 1.000000
56 CAGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTACGAGCCT 1.000000
57 CAGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTACGAGTCT 8.000000
58 CAGIAGTGGAATCAGCCGCTIACGGTGAGTGATAIGGGATCAIACGGCIGCDAGTCAGTICCIAGGTTCGAGCCT 4.000000
END LIST_SUMMARY

7.2.3 UDAC study

A full description of the UDAC study can be found in chapter 4, section 4.4.2.

7.2.4 DNA extraction and genotyping of variants in the UDAC study

DNA was extracted using the salting out methods described in section 2.2. Restriction fragment length polymorphism analysis (RFLP) oligonucleotides, PCR and digest conditions are detailed in Table 2.1 of chapter 2 (section 2.2.3). RFLP products were visualised using MADGE as described in section 2.2.4.2. All Taqman® assays are listed in Table 2.3 along with descriptions of Taqman® genotyping technique (section 2.2.6).

7.2.5 Measurement of sPLA2 IIA mass levels in Serum

Serum sPLA2 IIA mass levels were kindly measured by Olov Wiklund's group (Gothenburg Sweden), using a commercially available ELISA kit (Cayman Chemical company, Ann Arbor, MI). The assay was based on a double-antibody 'sandwich' ELISA, and assay conditions were as described by the manufacturer. 96 well plates coated with monoclonal antibodies specific to sPLA2 IIA were supplied. A 1 in 20 dilution of serum was made in buffer supplied with the ELISA kit and 100μL of sample added to each well. After the addition of the serum sample, an acetylcholinesterase conjugate (AChE:Fab) was applied to each well, which selectively binds a different epitope on the sPLA2 molecule. After washing away unbound antibody, Ellman's reagent (AChE substrate) was added and metabolised to form a coloured product which absorbs at 412nm. The intensity of colour is directly proportionate to the concentration of bound sPLA2 IIA. Intra- and inter- assay coefficients of variation (CV) were 6.0% and 10.3%, respectively. Secretory PLA2 IIA is an acute phase reactant (Vadas et al., 1993; Vadas et al., 1992) whose levels can increase greatly in those patients with underlying inflammation. Therefore all analysis was restricted to those patients with serum sPLA2 IIA levels within 3 standard deviations of the mean level.

7.2.6 Statistical analysis

Genotype information was initially collated on an EXCEL spreadsheet. All statistical analysis was performed by our Statisticians Jackie Cooper and Fotios Drenos as well as myself. The Hardy-Weinberg equilibrium and the linkage disequilibrium (D') of tSNPs were assessed using *THESIAS* (Tregouet et al., 2002; Tregouet et al., 2004). All analyses were performed on normally-distributed data after appropriate transformation (log or square

root). Parametric or non-parametric (Kruskal-Wallis) analysis of variance was used, when appropriate, to compare the changes of the continuous variables across the SNPs categories. Multiple regression was used to calculate the adjusted R-square for the proportion of the variance explained by the model. For the categorical variables Pearson's chi-square or Fisher's exact tests were used depending on the expected values of each category in the two-way table. Adjusted p values were obtained from analysis of covariance for continuous data and logistic regression for categorical data. Haplotypes were inferred using both *THESIAS* (Tregouet et al., 2002; Tregouet et al., 2004) and PHASE v2 (Stephens et al., 2001) excluding individuals with missing values. The haplotypic pair for each subject was calculated by PHASE (Stephens et al., 2001) and only the haplotypes with frequencies > 5% were used for further analysis. Haplotype additivity was tested using *THESIAS*. Because of multiple testing the significance level was taken as $p<0.01$, instead of an inappropriately conservative Bonferroni-like adjustment of the p-values (Rothman, 1990; Perneger, 1999).

7.3 Results for *PLA2G2A* analysis

7.3.1 Baseline characteristics

Table 7.1 summarises the baseline characteristics of the Caucasian men and women with T2DM, in relation to the presence and absence of CHD. Those individuals with CHD were significantly older and had a longer duration of diabetes. As with previous data relating to the UDAC study (see chapter 4), the higher usage of Statins, ACE inhibitors and aspirin potentially explains why those individuals with CHD had lower diastolic blood pressure, LDL cholesterol, and total cholesterol levels, compared to CHD-free men and women.

7.3.2 Serum sPLA2 measures

Serum sPLA2 IIA levels were not found to be significantly different by CHD status (Table 7.1, p=0.12). However, compared to men, women had significantly higher sPLA2 IIA levels (3.94ng/ml and 5.96ng/ml, respectively, p<0.0001). In the men, sPLA2 IIA levels were significantly negatively correlated with % total antioxidant status (%TAOS) and HDL, independent of age, CHD status, statin use and CRP levels (Table 7.2). Secretory PLA2 IIA levels were also positively correlated with CRP after adjustment for age, CHD status and statin use (Table 7.2).

In women, the proportion of CHD events significantly correlated with sPLA2 IIA levels, even after adjustment for age, statin use and CRP (Table 7.3), with women in the top two tertiles having statistically significantly higher CHD risk than those in the bottom tertile, [Odds Ratio (OR) of top two tertiles versus bottom tertile = 2.50 (95%CI (1.13-5.53) p=0.024 unadjusted and 4.82 (1.79-13.0) p=0.002 after adjustment)]. Although TAOS was negatively correlated with $sPLA_2IIa$ (p<0.009) this did not remain statistically significant after adjustment (Table 7.3).

Table 7.1 Baseline characteristics of Caucasian patients with T2DM from UDACS (±1SD)

Trait	No CHD N=383	CHD** N=136	P-value
Age (yrs)	65.5 (11.3)	69.5 (9.7)	0.0003
BMI (kg/m^2)*	29.2 (5.5)	29.5 (4.7)	0.67
HbA1c (%) *	7.7 (1.7)	7.5 (1.5)	0.27
Glucose (mmol/l)*	10.02 (4.40)	9.58 (4.25)	0.31
Cholesterol (mmol/l)	5.19 (1.07)	4.71 (1.12)	<0.0001
LDL (mmol/l)†	2.81 (0.93)	2.32 (0.89)	<0.0001
HDL (mmol/l)*	1.30 (0.38)	1.23 (0.37)	0.06
TG (mmol/l)*	1.90 (1.06)	1.92 (1.07)	0.84
SBP (mmHg)*	141.5 (20.6)	140.0 (20.9)	0.47
DBP (mmHg)	81.2 (11.4)	78.4 (10.0)	0.01
Duration of diabetes (yrs)‡	8 [4-16]	11 [6-17]	0.005
Gender (% male)	57.2% (219)	66.2% (90)	0.07
Smoking (% current)	17.0% (64)	12.0% (16)	0.18
TAOS (%)‡	44.9 [36.7-52.5]	42.9 [34.1-50.7]	0.13
Ox-LDL/LDL (U/mmol) *	16.8 (7.8)	18.6 (10.3)	0.08
PPD/MPD (nm) §	0.991 (0.016)	0.993 (0.018)	0.35
sdLDL (%) ‡	71.9 [58.5-81.4]	71.5 [54.9-80]	0.74
CRP (mg/L)*	1.66 (1.42)	1.77 (1.59)	0.49
sPLA2 IIA (ng/ml)*			
Total	3.08 (2.20)	3.45 (2.62)	0.12
Women	3.81 (2.66)	4.47 (2.89)	0.17
Men	2.62 (1.81)	3.02 (2.36)	0.12
Statin (%)	23.0	60.0	<0.0001
ACE I (%)	26.5	38.9	0.003
Aspirin (%)	21.6	43.4	<0.0001

*log-transformed
† square root transformed
‡median [IQR]
§peak partial diameter LDL/mean particle diameter LDL
** N=7 had missing CHD data

Table 7.2: Distribution of CHD and Diabetes risk factors (SD) according to sPLA2 IIA tertiles in Caucasian men with T2DM

	Tertile of sPLA2 IIA (ng/ml)			P-value for ANOVA		
	1	2	3	Unadjusted	Adjusted*	Adjusted†
	<1.95	1.95-3.30	>3.30			
	N=107	N=102	N=104			
TAOS (%)‖	48.6	42.2	41.2	0.0002	0.04	0.003
	[38.6-56]	[31.6-51.2]	[35-47.6]			
BMI (kg/m^2)‡	29.0 (5.0)	29.6 5.2)	29.4 (5.1)	0.65	0.29	0.68
LDL (mmol/l)§	2.56 (1.07)	2.67 (0.96)	2.56 (0.83)	0.69	0.54	0.36
HDL (mmol/l)‡	1.25 (0.37)	1.20 (0.33)	1.11 (0.28)	0.009	0.003	0.006
Ox-LDL/LDL (U/mmol)‡	18.9 (9.7)	15.1 (7.1)	18.7 (8.8)	0.01	0.01	0.01
sdLDL (%)‖	65.9	74.7	71.7	0.05	0.65	0.26
	[50.1-77.6]	[62.7-81.4]	[59.0-82.5]			
PPD/MPD (nm) #	0.990 (0.017)	0.992 (0.015)	0.991(0.017)	0.76	0.79	0.78
Cholesterol (mmol/l)	5.04 (1.22)	4.90 (1.03)	4.73 (1.03)	0.12	0.27	0.25
Smokers (%)	13.5 (14)	15.0 (15)	19.4 (20)	0.48	0.31	0.48
CRP (mg/l)	1.32 (1.02)	1.49 (1.32)	2.04 (1.72)	0.0007	0.0004	-
CHD (%)	25.0 (26)	29.4 (30)	33.0 (34)	0.45	0.68	0.91
Odds Ratio (95% CI)	1.00	1.25	1.48	0.21	0.39	0.66
		(0.68-2.31)	(0.81-2.71)			
HbA1c (%)‡	7.83 (1.70)	7.24 (1.47)	7.58 (1.54)	0.03	0.03	0.08
Glucose (mmol/l)‡	10.41 (4.40)	8.99 (4.07)	9.82 (4.32)	0.06	0.08	0.10

*adjusted for age, CAD status and statin use. † adjusted for age, CAD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.3: Distribution of the Coronary Heart Disease and Diabetes risk factors (SD) according to sPLA2 IIA tertiles in Caucasian women with T2DM

	Tertiles of sPLA2 IIA ng/ml			P-value for ANOVA		
	1	2	3	Unadjusted	Adjusted*	Adjusted†
	<2.82	2.82-4.66	>4.66			
	N=72	N=69	N=72			
TAOS (%)‖	49.4	44.0	41.2	0.009	0.06	0.65
	[37.9-56.5]	[35.4-49.5]	[31.8-48.7]			
BMI (kg/m^2)‡	29.3 (5.9)	28.7 (5.7)	29.9 (6.3)	0.44	0.33	0.72
LDL (mmol/l)§	2.83 (0.90)	2.84 (1.04)	2.74 (0.77)	0.76	0.51	0.50
HDL (mmol/l)‡	1.47 (0.40)	1.42 (0.35)	1.43 (0.44)	0.78	0.70	0.77
Ox-LDL/LDL (U/mmol)‡	18.5 (6.3)	15.8 (7.8)	15.8 (9.4)	0.18	0.24	0.25
sdLDL (%)‖	68.2	74.1	75.0	0.03	0.25	0.07
	[55.7-76.2]	[61.7-82.4]	[58.4-84.5]			
PPD/MPD (nm)#	0.993 (0.017)	0.992(0.019)	0.992 (0.013)	0.96	0.97	0.82
Cholesterol (mmol/l)	5.26 (1.04)	5.38 (1.24)	5.29 (0.87)	0.81	0.63	0.54
Smokers (%)	12.7 (9)	13.2 (9)	18.3 (13)	0.58	0.46	0.42
CRP (mg/l)‡	1.58 (1.32)	1.66 (1.59)	2.34 (1.96)	0.02	0.02	-
CAD (%)	12.7 (9)	31.3 (21)	22.2 (16)	0.03	0.01	0.002
Odds Ratio	1.00	3.14	1.97	0.17	0.06	0.02
(95% CI)		(1.32-7.50)	(0.81-4.81)			
HbA1c (%)‡	7.50 (1.73)	7.88 (1.63)	8.06 (1.58)	0.12	0.03	0.08
Glucose (mmol/l)‡	9.94 (4.49)	9.54 (4.16)	10.95 (4.59)	0.16	0.15	0.36

*adjusted for age, CAD status and statin use. † adjusted for age, CAD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

7.3.3 Location and allele frequencies of tSNPs

The STRAM algorithm identified 6 tSNPs in the *PLA2G2A* gene region based on the PHASE output from the NIEHS EGP website (http://egp.gs.washington.edu/data/*PLA2G2A*/) and these were successfully genotyped in the UDAC study (highlighted SNPs in PHASE output can be seen in Fig. 7.3). All tSNPs did not deviate from Hardy-Weinberg equilibrium as measured by *THESIAS*. The locations of the chosen tSNPs (one in the promoter region, two silent variants in exons, two present in introns, and two variants 3' of exon 6) are shown schematically in Figure 7.5. Lewontin's D' for each individual tSNP was determined from the Haploview 3.2 program developed by the Broad institute, Cambridge USA (http://www.broad.mit.edu/mpg/haploview/) and is shown below the SNP map in Figure 7.5. The tSNPs show regions of strong LD across the gene that appear to fall into two LD blocks separated by a region of low LD. Table 7.4 shows the relevant rs number and minor allele frequency of the 6 chosen tSNPs in the UDACS sample.

7.3.4 Individual tagging SNP associations with intermediate phenotypes

The univariate analyses of the identified tSNPs with intermediate phenotypes are presented in Tables 7.5 to 7.10. Four out of the six tSNPs (-655T>C, 763C>G, 1983G>A, 5128T>G) showed strong associations with sPLA2 IIA levels only ($p<0.0001$), while with a fifth SNP (1022G>T) the association was less strong ($p=0.01$). In addition -655T>C showed strong association with LDL cholesterol levels ($p=0.007$). These SNPs were primarily identified for tagging purposes and were essentially chosen to cover the genetic variability of the entire gene region. Therefore the subsequent haplotype analysis was covered in greater detail.

Fig 7.5: Map of the *PLA2G2A* gene showing the selected tagging SNPs numbered from the start of exon 1. Haploview LD (D') of tSNPs is shown below. The darker boxes represent the stronger LD. The D' for any two SNPs is presented in the box representing their intersection. No number indicates a D' of 1.

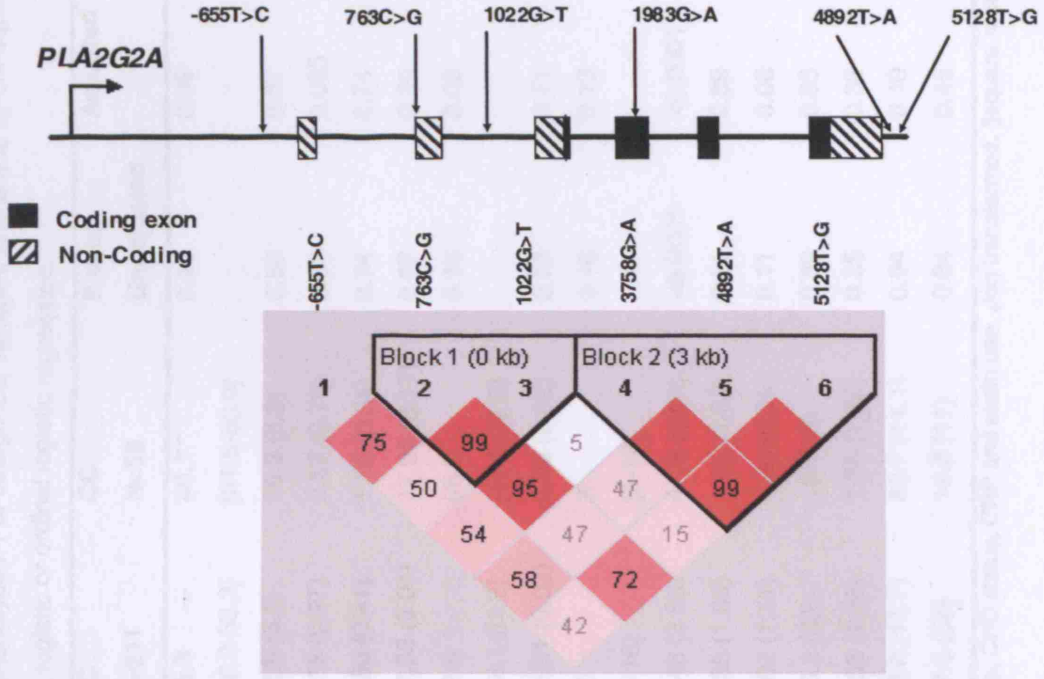


Table 7.4 Reference (rs) number and minor allele frequency (in UDACS Caucasians) of the chosen tSNPs in *PLA2G2A*

tSNP	rs number	Minor allele frequency (95%CI)
-655T>C	rs1774131	0.33 (0.30-0.36)
763C>G	rs11573156	0.23 (0.20-0.25)
1022G>T	rs3753827	0.45 (0.42-0.47)
1983G>A	rs2236771	0.11 (0.09-0.13)
4982T>A	rs876018	0.17 (0.15-0.19)
5128T>G	rs3767221	0.39 (0.36-0.42)

Table 7.5: T-655C SNP associations with intermediate phenotypes. For categorical variables analysis is by chi-squared test or Fisher's exact test. Adjustments made by analysis of covariance, logistic or ordinal logistic regression.

Trait	TT	TC	CC	P value	Adjusted*	Adjusted†
	N=229	N=211	N=59	Unadjusted		
TAOS (%)‖	44.0	45.3	40.1	0.21	0.09	0.10
	[34.9-52.1]	[36.2-53.3]	[31.5-50.0]			
BMI (kg/m^2)‡	29.5 (5.5)	28.9 (5.0)	29.3 (5.3)	0.50	0.67	0.72
LDL (mmol/l)§	2.82 (0.96)	2.59 (0.97)	2.57 (0.79)	0.03	0.005	0.007
HDL (mmol/l)‡	1.28 (0.36)	1.30 (0.41)	1.26 (0.34)	0.74	0.74	0.80
Ox-LDL/LDL (U/mmol)‡	17.11 (7.30)	17.33 (9.01)	17.96 (8.17)	0.87	0.79	0.72
sdLDL (%)‖	73.0	72.0	66.6	0.16	0.03	0.03
	[59.8-81.5]	[58.1-81.9]	[54.3-77.0]			
PPD/MPD (nm)#	0.992 (0.02)	0.991 (0.02)	0.99 (0.02)	0.73	0.71	0.78
Duration of diabetes (yrs)‖	9	10	7	0.16	0.13	0.17
	[4-17]	[5-16]	[5-12]			
$sPLA_2$-IIa(ng/ml)‡	2.68 (1.78)	3.48 (2.60)	4.15 (2.99)	<0.0001	<0.0001	<0.0001
TG (mmol/l)‡	1.94 (1.10)	1.85 (1.05)	1.97 (1.02)	0.61	0.89	0.92
Cholesterol (mmol/l)	5.18 (1.12)	5.02 (1.11)	4.88 (0.95)	0.11	0.06	0.07
Smokers (% current)	15.9 (36)	16.2 (33)	13.6 (8)	0.89	0.83	0.80
CRP (mg/l)‡	1.74 (1.50)	1.58 (1.36)	1.85 (1.78)	0.35	0.39	-
DBP (mmHg)	80.9 (10.1)	79.0 (11.7)	82.7 (11.1)	0.04	0.10	0.06
CHD (%)	27.6 (63)	27.6 (58)	18.6 (11)	0.34	0.45	0.50

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR].
peak particle diameter LDL/mean particle diameter LDL

Table 7.6: C763G SNP associations with intermediate phenotypes.

Trait	GG N=294	GC N=170	CC N=29	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.9 [35.4-53.3]	44.0 [35.4-53.3]	44.3 [37.2-51.2]	0.42	0.65	0.81
BMI (kg/m^2)‡	29.2 (5.5)	29. (5.1)	28.9 (4.4)	0.84	0.88	0.84
LDL (mmol/l)§	2.76 (0.99)	2.58 (0.89)	2.58 (0.87)	0.14	0.08	0.11
HDL (mmol/l)‡	1.29 (0.38)	1.30 (0.37)	1.20 (0.33)	0.33	0.48	0.51
Ox-LDL/LDL (U/mmol)‡	17.80 (8.13)	16.62 (8.15)	17.15 (9.07)	0.46	0.51	0.65
sdLDL (%)‖	72.5 [58.1-81.2]	72.0 [59.1-81.3]	64.0 [53.6-81.0]	0.43	0.39	0.21
PPD/MPD (nm)#	0.99 (0.02)	0.99(0.02)	0.99 (0.02)	0.34	0.40	0.35
Duration of diabetes (yrs)‖	9 [4-17]	9.5 [4-16]	7 [4-15.5]	0.69	0.68	0.64
$sPLA_2$-IIa (ng/ml)‡	2.59 (1.75)	4.24 (2.91)	4.75 (3.42)	<0.0001	<0.0001	<0.0001
TG (mmol/l)‡	1.90 (1.11)	1.86 (0.97)	2.00 (1.07)	0.78	0.66	0.66
Cholesterol (mmol/l)	5.12 (1.15)	4.98 (1.03)	4.96 (0.96)	0.34	0.30	0.31
Smokers (% current)	16.0 (46)	14.2 (24)	13.8 (4)	0.89	0.91	0.91
CRP (mg/l)‡	1.73 (1.49)	1.66 (1.48)	1.65(1.67)	0.87	0.93	-
DBP (mmHg)	80.9 (10.4)	79.4 (11.2)	84.0 (12.9)	0.08	0.10	0.13
CHD (%)	27.3 (80)	26.6 (45)	20.7 (6)	0.74	0.74	0.83

adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.7: G1022T SNP associations with intermediate phenotypes.

Trait	GG N=166	GT N=239	TT N=99	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.3 [35.9-52.1]	44.9 [35.4-52.3]	42.5 [32.3-50.2]	0.45	0.59	0.39
BMI (kg/m^2)‡	29.6 (5.3)	29.1 (5.7)	29.3 (4.6)	0.60	0.44	0.37
LDL (mmol/l)§	2.69 (0.88)	2.63 (1.00)	2.79 (0.89)	0.38	0.27	0.28
HDL (mmol/l)‡	1.28 (0.39)	1.28 (0.39)	1.30 (0.35)	0.86	0.92	0.98
Ox-LDL/LDL (U/mmol)‡	16.00 (7.73)	17.84 (8.40)	18.37 (8.27)	0.10	0.18	0.35
sdLDL (%)‖	71.7 [59.8-84.0]	71.6 [55.7-79.9]	74.4 [64.1-81.3]	0.42	0.63	0.61
PPD/MPD (nm)#	0.99 (0.02)	0.99 (0.02)	0.99(0.02)	0.45	0.53	0.53
Duration of diabetes (yrs)‖	9 [4-17]	9 [5-16]	8 [4-14]	0.34	0.22	0.24
$sPLA_2$-IIa (ng/ml)‡	3.57 (2.87)	3.06 (2.09)	2.87 (1.95)	0.03	0.02	0.01
TG (mmol/l)‡	1.89 (1.09)	1.93 (1.09)	1.87 (0.98)	0.88	0.94	0.93
Cholesterol (mmol/l)	5.06 (1.05)	5.05 (1.17)	5.10 (1.02)	0.93	0.88	0.95
Smokers (% current)	14.0 (23)	15.7 (37)	19.0 (18)	0.58	0.69	0.76
CRP (mg/l)‡	1.74 (1.49)	1.70 (1.51)	1.63 (1.37)	0.85	0.89	-
DBP (mmHg)	81.3 (11.5)	80.0 (10.9)	79.7 (10.0)	0.39	0.35	0.28
CHD (%)	27.9 (46)	25.1 (60)	29.6 (29)	0.66	0.52	0.54

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.8: G1983A SNP associations with intermediate phenotypes

Trait	GG N=399	GA N=82	AA N=13	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.0 [35.3-52.1]	45.6 [35.2-53.8]	45.6 [39.2-50.3]	0.69	0.58	0.51
BMI (kg/m^2)‡	29.3 (5.2)	28.5 (5.1)	32.1 (7.6)	0.07	0.05	0.04
LDL (mmol/l)§	2.69 (0.94)	2.55 (0.97)	3.23 (1.22)	0.07	0.17	0.19
HDL (mmol/l)‡	1.29 (0.37)	1.28 (0.40)	1.28 (0.40)	0.99	0.77	0.91
Ox-LDL/LDL (U/mmol)‡	17.10 (8.19)	19.13 (9.07)	16.60 (6.05)	0.30	0.44	0.54
sdLDL (%)‖	72.8 [58.5-81.6]	69.4 [50.3-77.6]	75.8 [69.9-81.0]	0.09	0.15	0.16
PPD/MPD (nm)#	0.99 (0.02)	0.99 (0.01)	0.99 (0.02)	0.70	0.73	0.88
Duration of diabetes (yrs)‖	9 [4-16]	10.5 [5-19]	12 [7-13]	0.47	0.55	0.43
$sPLA_2$-IIa (ng/ml)‡	3.36 (2.40)	2.54 (1.92)	2.33 (1.34)	0.002	0.0002	0.0001
TG (mmol/l)‡	1.86 (1.02)	2.10 (1.26)	1.92 (1.04)	0.21	0.16	0.24
Cholesterol (mmol/l)	5.06 (1.07)	5.02 (1.22)	5.61 (1.39)	0.19	0.29	0.30
Smokers (% current)	15.4 (60)	16.1 (13)	0 (0)	0.36	0.70	0.58
CRP (mg/l)‡	1.66 (1.45)	1.74 (1.47)	1.94 (1.97)	0.74	0.71	-
DBP (mmHg)	80.3 (11.0)	80.3 (10.3)	88.0 (8.4)	0.04	0.07	0.05
CHD (%)	26.7 (106)	30.5 (25)	15.4 (2)	0.54	0.91	0.94

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.9: T4982A SNP associations with intermediate phenotypes

Trait	AA	AT	TT	P value	Adjusted*	Adjusted†
	N=347	N=144	N=12	Unadjusted		
TAOS (%)‖	43.5	45.9	38.2	0.06	0.05	0.05
	[34.3-52.1]	[38.6-51.1]	[27.6-41.2]			
BMI (kg/m^2)‡	29.5 (5.2)	29.1 (5.7)	29.4 (5.4)	0.79	0.66	0.72
LDL (mmol/l)§	2.68 (0.96)	2.70 (0.92)	3.25 (0.74)	0.14	0.25	0.24
HDL (mmol/l)‡	1.28 (0.38)	1.30 (0.38)	1.25 (0.21)	0.87	0.66	0.62
Ox-LDL/LDL (U/mmol)‡	16.94 (8.47)	18.18 (7.26)	12.44 (2.26)	0.13	0.19	0.16
sdLDL (%)‖	71.1	73.7	78.0	0.83	0.58	0.53
	[57.7-81.0]	[56.0-81.5]	[54.3-79.4]			
PPD/MPD (nm)#	0.992 (0.017)	0.992 (0.015)	0.996 (0.016)	0.83	0.82	0.80
Duration of diabetes (yrs)‖	9	9	9	0.65	0.80	0.79
	[4-16]	[4.5-17]	[5.5-22]			
$sPLA_2$-IIa (ng/ml)‡	3.13 (2.26)	3.21 (2.36)	3.70 (1.75)	0.70	0.82	0.68
TG (mmol/l)‡	1.92 (1.04)	1.89 (1.15)	1.70 (0.91)	0.74	0.83	0.91
Cholesterol (mmol/l)	5.05 (1.12)	5.11 (1.08)	5.46 (0.81)	0.43	0.65	0.62
Smokers (% current)	14.7 (50)	18.4 (26)	16.7 (2)	0.53	0.64	0.72
CRP (mg/l)‡	1.76 (1.47)	1.54 (1.41)	1.49 (1.90)	0.27	0.21	-
DBP (mmHg)	80.8 (11.4)	79.4 (10.0)	80.8 (8.4)	0.39	0.38	0.34
CHD (%)	25.4 (88)	30.0 (43)	16.7 (2)	0.49	0.18	0.16

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed .‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.10: T5128G SNP associations with intermediate phenotypes

Trait	TT N=194	TG N=222	GG N=87	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.3 [35.4-52.1]	44.8 [35.4-51.0]	43.9 [35.2-53.3]	0.93	0.81	0.71
BMI (kg/m^2)‡	28.9 (5.1)	29.4 (5.4)	29.7 (5.8)	0.47	0.57	0.54
LDL (mmol/l)§	2.72 (0.83)	2.62 (0.97)	2.81 (1.12)	0.28	0.43	0.44
HDL (mmol/l)‡	1.27 (0.35)	1.30 (0.38)	1.30 (0.42)	0.79	0.79	0.70
Ox-LDL/LDL (U/mmol)‡	17.55 (7.38)	17.06 (8.85)	17.45 (8.01)	0.88	0.71	0.84
sdLDL (%)‖	72.5 [55.2-79.4]	72.9 [60.4-83.6]	69.9 [54.2-78.2]	0.18	0.12	0.16
PPD/MPD (nm)#	0.99 (0.02)	0.99 (0.02)	0.99 (0.02)	0.13	0.17	0.22
Duration of diabetes (yrs)‖	7.5 [4-14]	9 [5-17]	9 [5-17]	0.09	0.07	0.08
$sPLA_2$-IIa (ng/ml)‡	3.94 (3.01)	3.03 (2.08)	2.17 (1.15)	<0.0001	<0.0001	<0.0001
TG (mmol/l)‡	1.88 (1.04)	1.89 (1.03)	1.96 (1.19)	0.85	0.80	0.92
Cholesterol (mmol/l)	5.08 (0.99)	5.02 (1.11)	5.17 (1.26)	0.57	0.58	0.55
Smokers (% current)	16.5 (31)	14.9 (33)	16.5 (14)	0.90	0.99	0.99
CRP (mg/l)‡	1.67 (1.56)	1.68 (1.41)	1.80 (1.49)	0.79	0.82	-
DBP (mmHg)	79.7 (11.4)	80.2 (10.2)	82.8 (11.4)	0.09	0.10	0.07
CHD (%)	25.0 (48)	29.7 (66)	23.0 (20)	0.38	0.55	0.39

*adjusted for age, CHD status and statin use. †adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

7.3.5 Haplotype frequencies in UDACS

Of the potential 64 haplotypes defined by 6 tSNPs, 23 inferred haplotypes were observed in the sample. Of these haplotypes, 6 occurred at frequencies >5% and accounted for 68% of the genetic variation within the gene. Twelve tSNP haplotypes occurred at frequencies between 1% and 5%, and 5 occurred at frequencies <1% (Table 7.11). The common inferred haplotype frequencies identified in the PDR 90 population differed from those Haplotype frequencies inferred in the UDACS sample (see Fig. 7.3).

Table 7.11: Inferred haplotype frequencies in UDACS. tSNP order from left to right is: -655, 763, 1022, 1983, 4982, 5128

Haplotype name	Haplotype combination	Frequency in UDACS
H1	CCGGAT	0.142
H2	TGGGAG	0.132
H3	TGTGAT	0.131
H4	TGTGTT	0.113
H5	TGTGAG	0.080
H6	TGGGAT	0.077
	TGGAAG	0.050
	CGTGAT	0.0442
	TGTAAG	0.043
	TGGGTT	0.031
	CGGGAT	0.031
	TCGGAT	0.0231
	CGGGAG	0.023
	CCGGAG	0.0153
	CGGAAG	0.015
	CCGGTT	0.012
	CGTGAG	0.0112
	CGTGTT	0.010
	TCGGTT	0.006
	TCGGAG	0.0056
	CGTAAG	0.0036
	TCGAAG	0.0009
	CCTGAT	0.0008

7.3.6 Haplotype associations with sPLA2 IIA levels and intermediate phenotypes

Overall, haplotypic variation in the *PLA2G2A* gene was associated with significant effects on sPLA2 IIA mass levels in the UDAC study ($p<0.0001$). The most frequently occurring haplotype, H1 (CCGGAT), was associated with 53% higher sPLA2 IIA levels [2.36 (1.58-3.51) ng/ml] when compared against all the other 5 haplotypes combined ($p<0.00001$). The sPLA2 IIA mass levels did not significantly differ from each other for the other five haplotypes ($p=0.34$, see table 7.12). Haplotypic variation in the *PLA2G2A* gene explained 6.3% of the variance in sPLA2 IIA levels. However, when considering other intermediate phenotypes in the UDAC study, there was no significant association of any haplotype with these traits (Table 7.13 A and B).

Table 7.12 *PLA2G2A* tSNP haplotypes which occur at frequencies >5% and their association with sPLA2 IIA levels. *Haplotypic mean obtained from log transformed data. Overall significance for haplotype variation $p<0.0001$

Haplotype	Frequency %	sPLA2 IIA levels ng/ml (95%CI)*	P-value compared to CCGGAT
H1: CCGGAT	14.2	2.36 (1.58-3.51)	-
H2: TGGGAG	13.3	1.40 (1.09-1.81)	<0.0001
H3: TGTGAT	13.1	1.55 (1.17-2.06)	<0.002
H4: TGTGTT	11.3	1.62 (1.24-2.12)	<0.007
H5: TGTGAG	8.0	1.42 (1.02-1.96)	<0.001
H6: TGGGAT	7.7	1.75 (1.14-2.06)	0.65

Table 7.13 A: *PLA2G2A* tSNP haplotypes which occur at frequencies >5% and their association with intermediate phenotypes (haplotypic means and 95%CI).

Haplotype	LDL (mmol/l)§	P value compared to CCGGAT	Small dense LDL (%)	P value compared to CCGGAT	oxLDL/LDL (U/mmol)‡	P value compared to CCGGAT
H1: CCGGAT	1.28 (1.21-1.36)	-	33.7 (32.2-35.1)	-	9.09 (8.17-10.12)	-
H2: TGGGAG	1.40 (1.24-1.56)	0.25	37.2 (33.7-40.6)	0.09	7.33 (6.00-8.96)	0.09
H3: TGTGAT	1.30 (1.13-1.48)	0.87	38.1 (33.9-42.4)	0.06	9.36 (7.41-11.82)	0.83
H4: TGTGTT	1.39 (1.20-1.58)	0.34	36.4 (32.9-39.9)	0.18	8.69 (6.79-11.11)	0.75
H5: TGTGAG	1.33 (1.13-1.56)	0.68	33.3 (28.1-38.4)	0.89	8.06 (6.12-10.61)	0.44
H6: TGGGAT	1.50 (1.22-1.81)	0.17	33.6 (26.9-40.3)	0.98	6.70 (4.72-9.53)	0.13

Haplotype	HDL(mmol/l)‡	P value compared to CCGGAT	% TAOS	P value compared to CCGGAT	UKPDS risk score	P value compared to CCGGAT
H1: CCGGAT	0.65 (0.62-0.69)	-	21.18 (20.0-22.4)	-	12.2 (10.7-13.9)	-
H2: TGGGAG	0.65 (0.60-0.71)	0.97	21.28 (19.3-23.2)	0.32	14.1 (11.2-17.6)	0.32
H3: TGTGAT	0.61 (0.55-0.68)	0.27	20.56 (18.4-22.7)	0.63	11.9 (8.4-16.9)	0.90
H4: TGTGTT	0.66 (0.59-0.73)	0.88	22.03 (19.7-24.4)	0.56	14.3 (10.8-18.9)	0.36
H5: TGTGAG	0.65 (0.56-0.75)	0.97	19.25 (16.1-22.4)	0.28	11.4 (7.47-17.3)	0.76
H6: TGGGAT	0.62 (0.50-0.78)	0.72	23.70 (19.0-28.4)	0.32	12.8 (7.2-22.7)	0.88

‡log-transformed. §square root transformed.

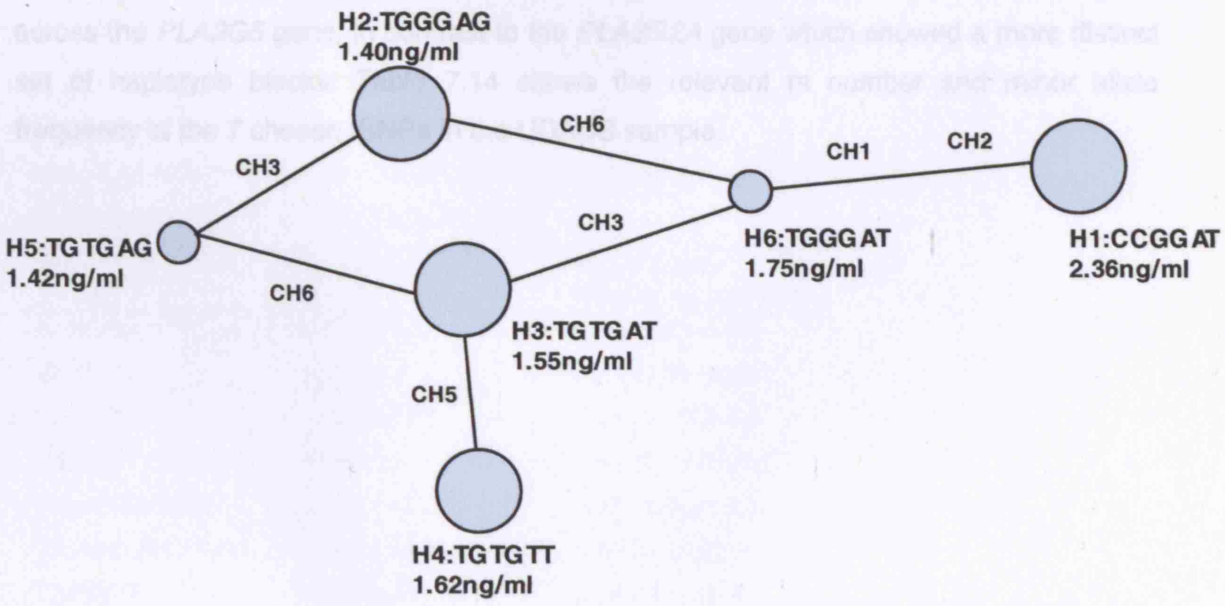
Table 7.13 B: p values for other associations tested

Haplotype	P value for TG	P value for Cholesterol
H1: CCGGAT	-	-
H2: TGGGAG	0.63	0.27
H3: TGTGAT	0.90	0.97
H4: TGTGTT	0.57	0.44
H5: TGTGAG	0.23	0.33
H6: TGGGAT	0.22	0.93

7.3.7 Evolutionary relationship of Haplotypes

To investigate whether a functional SNP was associated with the sPLA2 IIA raising effects of haplotype H1, a cladogram, representing the evolutionary relationship of the haplotypes was drawn up using the website http://fluxus-engineering.com (Fig.7.6). H1 is separated from H6 by two changes in the T-655C and C763G tSNPs; while H2 and H3 are separated from H6 by a single change each (T5128G and G1022T respectively). H5 could be derived from either H2 or H3 by a single change (G1022T and T5128G respectively), while H4 is separated from H3 by a single change (T4982A). Therefore, no single SNP could distinguish H1 from the other common haplotypes, and suggests that none of the selected tSNPs are themselves functional.

Fig 7.6: Tagging SNP Cladogram for *PLA2G2A*. The diameters of the circles represent the observed frequencies of the inferred haplotypes. Text along the line represents the changes in tSNPs that have occurred i.e. CH6 represents a change to tSNP 6. When more than one change has occurred, the order on the figure becomes arbitrary.



7.4 Results for *PLA2G5* analysis

7.4.1 Baseline characteristics

Baseline characteristics for the UDAC study as a whole are shown in table 7.1 and described in section 7.3.1. Unfortunately no measures were available for sPLA2 V mass levels.

7.4.2 Location and allele frequencies of tSNPs

Using the STRAM algorithm, we identified 7 tSNPs in the *PLA2G5* gene region based on the PHASE output from the NIEHS EGP website (http://egp.gs.washington.edu/data/*PLA2G5*/) and these were successfully genotyped in the UDAC study (Those chosen SNPs are highlighted red in the PHASE output seen in Fig. 7.4). None of these tSNPs deviated from Hardy-Weinberg equilibrium as tested by *THESIAS*. The locations of the chosen tSNPs (three in the promoter region, three within introns, and one variant 3' of exon 5) are shown schematically in Figure 7.7. Lewontin's D' for each individual tSNP was determined from the Haploview 3.2 program (Broad institute, USA) and is shown below the SNP map in Figure 7.7. The tSNPs showed strong LD across the *PLA2G5* gene, in contrast to the *PLA2G2A* gene which showed a more distinct set of haplotype blocks. Table 7.14 shows the relevant rs number and minor allele frequency of the 7 chosen tSNPs in the UDACS sample.

Fig. 7.7: Map of the *PLA2G5* gene showing the selected tagging SNPs numbered from the start of exon 1. Haploview LD (D') of tSNPs is shown below. The darker boxes represent the stronger LD. The D' for any two SNPs is presented in the box representing their intersection. No number indicates a D' of 1.

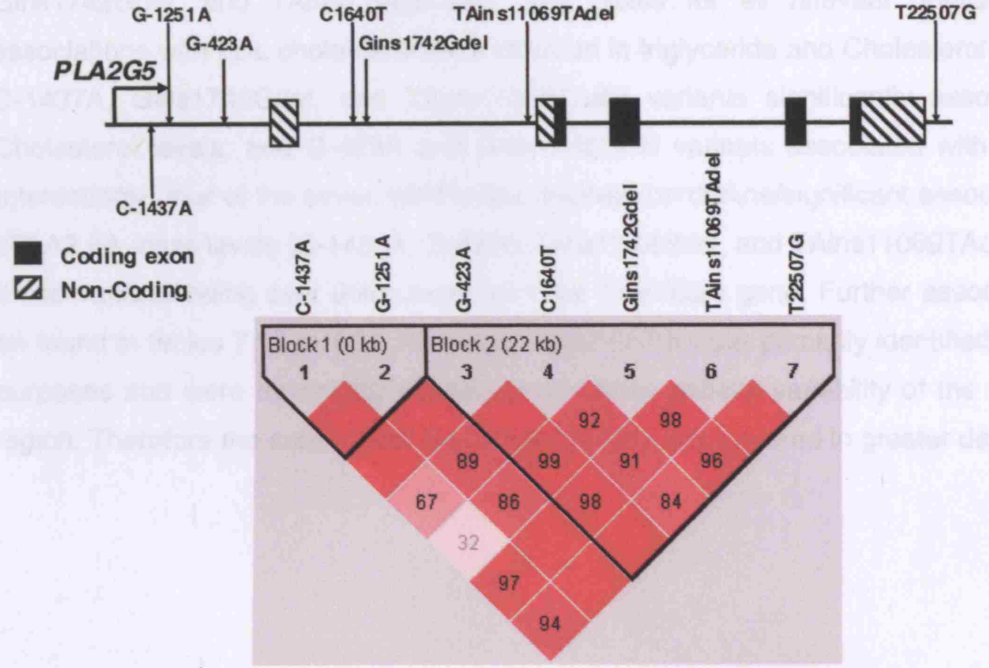


Table 7.14 Reference (rs) number and minor allele frequency (in UDACS Caucasians) of the chosen tSNPs in *PLA2G5*

tSNP	rs number	Minor allele frequency (95%CI)
C-1437A	rs11573185	0.45 (0.43-0.48)
G-1251A	rs2148911	0.07 (0.05-0.08)
G-423A	rs11573191	0.18 (0.16-0.19)
C1640T	rs640022	0.15 (0.13-0.16)
Gins1742Gdel	rs11573203	0.28 (0.26-0.30)
TAins11069TAdel	rs11573248	0.36 (0.34-0.39)
T22507G	rs622450	0.14 (0.12-0.15)

7.4.3 Single SNP associations with intermediate phenotypes

The univariate analyses of the identified tSNPs with intermediate phenotypes are presented in Tables 7.15 to 7.21. Of particular interest was the observation that four of the tSNPs showed a significant association with LDL levels (C-1437A, G-423A, Gins1742Gdel, and TAins11069TAdel, see tables for all relevant p-values). These associations with LDL cholesterol were mirrored in triglyceride and Cholesterol levels; with C-1437A, Gins1742Gdel, and TAins11069TAdel variants significantly associated with Cholesterol levels; and G-423A and Gins1742Gdel variants associated with TG levels. Interestingly, four of the seven tSNPs also showed borderline/significant associations with sPLA2 IIA mass levels (C-1437A, G-423A, Gins1742Gdel, and TAins11069TAdel), despite these variants being over 90kb away from the *PLA2G2A* gene. Further associations can be found in tables 7.15 to 7.21. However, these SNPs were primarily identified for tagging purposes and were essentially chosen to cover the genetic variability of the entire gene region. Therefore the subsequent haplotype analysis was covered in greater detail.

Table 7.15: C-1437A SNP associations with intermediate phenotypes. For categorical variables analysis is by chi-squared test or Fisher's exact test. Adjustments made by analysis of covariance, logistic or ordinal logistic regression.

	CC	CA	AA	P value	Adjusted*	Adjusted†
	N=105	N=244	N=155	Unadjusted		
TAOS (%)‖	45.5	44	44.8	0.29	0.47	0.67
	[36.5-53.9]	[34.5-50.3]	[35.6 -52.1]			
BMI (kg/m^2)‡	29.9 (6.1)	29.4 (5.4)	28.8 (4.6)	0.29	0.22	0.31
LDL (mmol/l)§	2.85 (0.96)	2.70 (1.01)	2.58 (0.81)	0.08	0.01	0.02
HDL (mmol/l)‡	1.30 (0.39)	1.29 (0.39)	1.27 (0.34)	0.74	0.66	0.66
Ox-LDL/LDL (U/mmol)‡	17.22 (5.82)	16.49 (8.39)	18.57 (8.73)	0.14	0.09	0.09
sdLDL (%)‖	71.3	71.9	72.9	0.93	0.80	0.62
	[56.4-82.9]	[58.1-81.2]	[57.4-79.6]			
PPD/MPD (nm)#	0.991 (0.016)	0.994 (0.015)	0.988 (0.019)	0.02	0.03	0.03
Duration of diabetes (yrs)‖	9	9	8	0.73	0.73	0.63
	[5-17]	[4-17]	[5-15]			
$sPLA_2$-IIa(ng/ml)‡	2.76 (2.00)	3.22 (2.27)	3.32 (2.41)	0.10	0.06	0.04
TG (mmol/l)‡	2.06 (1.28)	1.84 (0.98)	1.91 (1.03)	0.20	0.15	0.19
Cholesterol (mmol/l)	5.30 (1.15)	5.09 (1.15)	4.91 (0.91)	0.02	0.004	0.006
Smokers (% current)	11.4 (12)	19.3 (46)	13.3 (20)	0.11	0.09	0.07
CRP (mg/l)‡	1.70 (1.51)	1.77 (1.53)	1.59 (1.39)	0.48	0.52	-
DBP (mmHg)	80.3 (11.5)	80.0 (10.5)	81.1 (11.2)	0.62	0.71	0.76
CHD (%)	29.8 (31)	28.4 (69)	20.0 (31)	0.11	0.19	0.30

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.16: G-1251A SNP associations with intermediate phenotypes.

	GG N=440	GA N=61	AA N=4	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.3 [35.3-51.8]	45.6 [34.5-51.1]	49.8 [44.5-56.4]	0.42	0.31	0.28
BMI (kg/m^2)‡	29.4 (5.3)	28.6 (5.3)	31.0 (10.2)	0.46	0.78	0.44
LDL (mmol/l)§	2.69 (0.96)	2.72 (0.88)	3.29 (0.70)	0.48	0.46	0.48
HDL (mmol/l)‡	1.29 (0.37)	1.27 (0.41)	1.08 (0.23)	0.47	0.54	0.58
Ox-LDL/LDL (U/mmol)‡	17.37 (8.32)	16.67 (7.29)	17.67 (3.69)	0.87	0.88	0.98
sdLDL (%)‖	72.1 [56.2-81.2]	70.9 [59.9-81.5]	78.2 [43.2-91.4]	0.79	0.96	0.94
PPD/MPD (nm)#	0.992 (0.017)	0.991 (0.013)	0.991 (0.003)	0.99	0.98	0.98
Duration of diabetes (yrs)‖	8 [4-16]	12 [6-19]	5 [1.5-7]	0.01	0.13	0.07
$sPLA_2$-IIa(ng/ml)‡	3.14 (2.20)	3.45 (3.11)	2.92 (1.74)	0.62	0.95	0.92
TG (mmol/l)‡	1.89 (1.05)	1.95 (1.15)	2.95 (1.05)	0.27	0.28	0.33
Cholesterol (mmol/l)	5.07 (1.09)	5.12 (1.13)	5.60 (1.10)	0.58	0.57	0.60
Smokers (% current)	16.2 (70)	13.6 (8)	0 (0)	0.85	0.61	0.50
CRP (mg/l)‡	1.67 (1.44)	1.93 (1.75)	1.72 (1.29)	0.51	0.48	-
DBP (mmHg)	80.5 (11.1)	78.4 (10.4)	88.8 (3.3)	0.12	0.22	0.15
CHD (%)	25.8 (113)	29.5 (18)	25.0 (1)	0.80	0.83	0.93

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.17: G-423A SNP associations with intermediate phenotypes.

	GG	GA	AA	P value	Adjusted*	Adjusted†
	N=347	N=146	N=14	Unadjusted		
TAOS (%)‖	44.8	43.4	50.4	0.48	0.36	0.31
	[35.3-51.2]	[34.5-51.7]	[37.9-58.6]			
BMI (kg/m^2)‡	29.0 (5.3)	30.0 (5.4)	29.9 (6.1)	0.15	0.16	0.20
LDL (mmol/l)§	2.62 (0.90)	2.88 (1.04)	2.79 (1.12)	0.02	0.01	0.02
HDL (mmol/l)‡	1.28 (0.37)	1.28 (0.39)	1.26 (0.32)	0.96	0.96	0.98
Ox-LDL/LDL (U/mmol)‡	17.56 (8.63)	16.56 (7.03)	19.0 (8.05)	0.49	0.61	0.47
sdLDL (%)‖	72.9	70.0	75.0	0.81	0.97	0.93
	[57.4-81.3]	[59.8-81.0]	[47.1-85.3]			
PPD/MPD (nm)#	0.991 (0.016)	0.993 (0.017)	0.992 (0.024)	0.56	0.59	0.64
Duration of diabetes (yrs)‖	9	8	8.5	0.20	0.30	0.32
	[5-16]	[3-14]	[4-18]			
$sPLA_2$-IIa(ng/ml)‡	3.36 (2.44)	2.90 (2.07)	1.93 (1.12)	0.004	0.004	0.002
TG (mmol/l)‡	1.88 (1.05)	1.90 (1.06)	2.80 (1.37)	0.03	0.03	0.03
Cholesterol (mmol/l)	5.00 (1.04)	5.25 (1.20)	5.19 (1.23)	0.06	0.04	0.06
Smokers (% current)	15.6 (53)	16.6 (24)	7.1 (1)	0.80	0.62	0.60
CRP (mg/l)‡	1.61 (1.42)	1.93 (1.61)	1.66 (1.47)	0.12	0.13	-
DBP (mmHg)	79.9 (10.9)	81.1 (11.9)	84.2 (8.3)	0.22	0.19	0.14
CHD (%)	15.4 (88)	27.6 (40)	42.9 (6)	0.33	0.34	0.30

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.18: C1640T SNP associations with intermediate phenotypes.

	CC N=356	CT N=130	TT N=15	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.8 [35.9-52.7]	43.5 [32.2-50.6]	42.4 [36.9-52.8]	0.50	0.40	0.43
BMI (kg/m^2)‡	29.3 (5.3)	29.4 (5.6)	26.7 (3.5)	0.14	0.18	0.10
LDL (mmol/l)§	2.72 (0.97)	2.61 (0.93)	2.83 (0.74)	0.48	0.62	0.52
HDL (mmol/l)‡	1.27 (0.35)	1.31 (0.43)	1.47 (0.59)	0.11	0.05	0.04
Ox-LDL/LDL (U/mmol)‡	17.76 (7.85)	16.20 (9.33)	16.67 (4.30)	0.32	0.26	0.54
sdLDL (%)‖	72.3 [57.4-81.0]	70.8 [55.8-81.4]	78.2 [68.9-83.6]	0.56	0.53	0.48
PPD/MPD (nm)#	0.991 (0.018)	0.993 (0.014)	0.994 (0.007)	0.74	0.75	0.71
Duration of diabetes (yrs)‖	9 [4-15.5]	9 [5-17]	11 [5-18]	0.55	0.44	0.34
$sPLA_2$-IIa(ng/ml)‡	3.07 (2.14)	3.35 (2.70)	3.76 (2.55)	0.32	0.72	0.95
TG (mmol/l)‡	1.93 (1.06)	1.83 (1.04)	2.14 (1.47)	0.49	0.68	0.59
Cholesterol (mmol/l)	5.08 (1.09)	5.01 (1.12)	5.56 (1.17)	0.19	0.16	0.15
Smokers (% current)	15.8 (55)	14.0 (18)	28.6 (4)	0.34	0.34	0.37
CRP (mg/l)‡	1.64 (1.48)	1.77 (1.41)	2.22 (1.66)	0.32	0.32	-
DBP (mmHg)	80.9 (10.8)	79.5 (11.6)	78.5 (7.6)	0.38	0.60	0.55
CHD (%)	24.9 (88)	31.5 (41)	20.0 (3)	0.31	0.44	0.67

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.19: Gins1742Gdel variant associations with intermediate phenotypes.

	Gins	InsG/delG	Gdel	P value	Adjusted*	Adjusted†
	N=261	N=190	N=39	Unadjusted		
TAOS (%)‖	44.9	43	42.3	0.95	0.66	0.62
	[35.1-52.1]	[35.3-51.2]	[35.2-56.6]			
BMI (kg/m^2)‡	28.7 (5.0)	30.1 (5.7)	29.6 (5.2)	0.03	0.03	0.02
LDL (mmol/l)§	2.58 (0.93)	2.80 (0.95)	2.92 (1.06)	0.02	0.007	0.01
HDL (mmol/l)‡	1.28 (0.39)	1.29 (0.37)	1.30 (0.33)	0.89	0.92	0.92
Ox-LDL/LDL (U/mmol)‡	18.10 (9.45)	16.69 (6.50)	17.02 (7.98)	0.33	0.40	0.23
sdLDL (%)‖	72.3	70.7	73.6	0.49	0.66	0.67
	[57.4-81.6]	[59.8-80]	[45.1-80.3]			
PPD/MPD (nm)#	0.990 (0.016)	0.993 (0.016)	0.992 (0.021)	0.27	0.28	0.37
Duration of diabetes (yrs)‖	10	8	9	0.09	0.13	0.13
	[5-16]	[4-15]	[4-17]			
$sPLA_2$-IIa(ng/ml)‡	3.39 (2.40)	3.05 (2.18)	2.33 (1.56)	0.006	0.008	0.007
TG (mmol/l)‡	1.86 (1.04)	1.88 (1.05)	2.55 (1.34)	0.004	0.004	0.003
Cholesterol (mmol/l)	4.97 (1.07)	5.15 (1.10)	5.48 (1.22)	0.01	0.01	0.01
Smokers (% current)	14.4 (37)	20.0 (37)	7.7 (3)	0.11	0.06	0.04
CRP (mg/l)‡	1.66 (1.45)	1.73 (1.52)	1.73 (1.40)	0.86	0.94	-
DBP (mmHg)	79.9 (10.5)	80.5 (11.8)	81.9 (10.6)	0.50	0.53	0.50
CHD (%)	25.0 (65)	28.6 (54)	28.2 (11)	0.68	0.71	0.75

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.20: TAins11069TAdel variant associations with intermediate phenotypes.

	TAins N=210	InsTA/delTA N=227	TAdel N=69	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44.7	43.4	46.2	0.27	0.17	0.10
	[35.4-52.8]	[33.1-50.3]	[37.2-52.5]			
BMI (kg/m^2)‡	29.6 (5.8)	29.3 (5.4)	28.6 (3.7)	0.44	0.42	0.55
LDL (mmol/l)§	2.84 (0.93)	2.64 (0.99)	2.46 (0.83)	0.007	0.0003	0.0005
HDL (mmol/l)‡	1.31 (0.39)	1.28 (0.38)	1.22 (0.33)	0.29	0.21	0.20
Ox-LDL/LDL (U/mmol)‡	16.65 (6.86)	17.24 (8.68)	19.30 (9.72)	0.20	0.11	0.13
sdLDL (%)‖	73.1	71.4	70	0.85	0.62	0.59
	[55.5-81.6]	[60.2-80.3]	[55.8-81.0]			
PPD/MPD (nm)#	0.993 (0.016)	0.992 (0.016)	0.987(0.018)	0.07	0.08	0.09
Duration of diabetes (yrs) ‖	9	8	10	0.91	0.81	0.91
	[4-16]	[4-17]	[5-16]			
$sPLA_2$-IIa(ng/ml)‡	3.01 (2.28)	3.17 (2.18)	3.78 (2.76)	0.08	0.05	0.02
TG (mmol/l)‡	2.05 (1.20)	1.82 (0.97)	1.74 (0.90)	0.03	0.05	0.06
Cholesterol (mmol/l)	5.30 (1.13)	4.98 (1.08)	4.71 (0.89)	<0.0001	<0.0001	<0.0001
Smokers (% current)	15.6 (32)	18.4 (41)	7.3 (5)	0.08	0.05	0.05
CRP (mg/l)‡	1.66 (1.42)	1.80 (1.58)	1.47 (1.30)	0.23	0.30	-
DBP (mmHg)	80.2 (10.7)	79.9 (10.9)	82.3 (12.2)	0.27	0.23	0.14
CHD (%)	29.7 (62)	24.8 (56)	21.7 (15)	0.33	0.50	0.67

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

Table 7.21: T22507G variant associations with intermediate phenotypes.

	TT N=381	TG N=110	GG N=11	P value Unadjusted	Adjusted*	Adjusted†
TAOS (%)‖	44 [35.2-52.1]	45.8 [35.7-50.8]	41.2 [33.6-56.9]	0.87	0.81	0.89
BMI (kg/m^2)‡	29.5 (5.1)	28.7 (5.9)	29.6 (6.9)	0.40	0.30	0.28
LDL (mmol/l)§	2.67 (0.94)	2.76 (1.03)	2.62 (0.84)	0.69	0.61	0.59
HDL (mmol/l)‡	1.28 (0.37)	1.30 (0.40)	1.41 (0.53)	0.49	0.61	0.55
Ox-LDL/LDL (U/mmol)‡	17.33 (8.38)	17.40 (7.45)	17.41 (3.41)	0.997	0.96	0.97
sdLDL (%)‖	71.9 [57.4-81.0]	72.8 [54.9-81.9]	73.7 [58.5-90.1]	0.86	0.80	0.79
PPD/MPD (nm)#	0.991 (0.017)	0.993 (0.014)	0.988 (0.018)	0.61	0.64	0.60
Duration of diabetes (yrs)‖	9 [4-17]	9 [6-14]	12 [6-27]	0.57	0.66	0.60
$sPLA_2$-IIa(ng/ml)‡	3.14 (2.29)	3.21 (2.25)	2.98 (2.37)	0.92	0.83	0.78
TG (mmol/l)‡	1.94 (1.07)	1.78 (0.97)	2.02 (1.72)	0.38	0.32	0.35
Cholesterol (mmol/l)	5.04 (1.10)	5.17 (1.13)	5.27 (1.03)	0.46	0.30	0.25
Smokers (% current)	14.9 (56)	18.7 (20)	9.1 (1)	0.61	0.56	0.66
CRP (mg/l)‡	1.74 (1.44)	1.53 (1.50)	1.27 (1.30)	0.23	0.43	-
DBP (mmHg)	80.9 (11.3)	78.8 (10.2)	79.1 (4.9)	0.20	0.12	0.09
CHD (%)	25.0 (95)	27.3 (30)	40.0 (4)	0.44	0.62	0.60

*adjusted for age, CHD status and statin use. † adjusted for age, CHD status, CRP and statin use. ‡log-transformed. §square root transformed. ‖median [IQR]. # peak particle diameter LDL/mean particle diameter LDL

7.4.4 Haplotype frequencies in UDACS

Of the potential 119 haplotypes defined by 7 tSNPs, 18 inferred haplotypes were observed in the sample. Of these haplotypes, 7 occurred at frequencies >5% and accounted for 92% of the genetic variation within the gene. Two tSNP haplotypes occurred at a frequency >1% (Table 7.22).

Table 7.22: Inferred haplotype frequencies in UDACS. tSNP order from left to right is: -1437, -1251, -423, 1640, 1742, 11609, and 22507

Haplotype name	Haplotype combination final	Frequency in UDACS
H1	AGGCGDT	0.359
H2	CGACDIT	0.169
H3	CGGCGIG	0.128
H4	AGGCDIT	0.094
H5	AGGCGIT	0.060
H6	CAGTGIT	0.059
H7	CGGTGIT	0.055
	AGGTGIT	0.032
	CGGCGIT	0.026
	CGGCDIT	0.003
	AGGCGIG	0.003
	CAGTGIG	0.002
	CGACDDT	0.002
	AGGTDIT	0.002
	CGGTGIG	0.002
	AGGTGDT	0.001
	CGGCGDG	0.001
	CAGCGIT	0.001

7.4.5 Associations of *PLA2G5* haplotypes with intermediate phenotypes

Tables 7.23 A and B show the associations of the common *PLA2G5* haplotypes with intermediate phenotypes. Associations were tested against the most commonly occurring haplotype H1, and a p value <0.01 was considered statistically significant. In single SNP analysis there were significant associations of several tSNPs with lipid measures. In Haplotype analysis, H2 was associated with significantly higher TG (29.8%), cholesterol (13.8%) and LDL levels (25.4%) compared to H1 ($p=0.01$, $p<0.001$, and $p<0.001$ respectively). In addition, H3 was associated with higher cholesterol levels (13.8%, $p=0.003$) compared to H1. Haplotype H4 was also associated with raised TG (44%, $p=0.003$) and cholesterol levels (19.0%, $p<0.001$), and a borderline significant association with LDL (22.1%, $p=0.017$). Haplotype H5 was significantly associated with raised TG levels (64.3%, $p=0.002$), and H7 was significantly associated with lowered oxLDL/LDL levels (32.2%, $p=0.01$). There were no significant associations of any of the haplotypes with sPLA2 IIA mass levels, HDL, %sdLDL, %TAOS, or UKPDS risk score

Table 7.23 A: Association of Intermediate phenotypes and common haplotypes.

Haplotype	sPLA2 IIA levels (ng/ml)‡	P value compared to AGGCGDT	TG (mmol/L)‡	P value compared to AGGCGDT	Cholesterol (mmol/L)	P value compared to AGGCGDT
H1: AGGCGDT	1.80 (1.54-2.10)	-	0.84 (0.75-0.93)	-	2.32 (2.23-2.42)	-
H2: CGACDIT	1.29 (1.03-1.62)	0.03	1.09 (0.92-1.29)	0.01	2.64 (2.50-2.79)	<0.001
H3: CGGCGIG	1.49 (1.13-1.97)	0.26	0.92 (0.79-1.08)	0.32	2.64 (2.47-2.82)	0.003
H4: AGGCDIT	1.52 (1.14-2.04)	0.33	1.21 (0.98-1.50)	0.003	2.76 (2.52-3.01)	<0.001
H5: AGGCGIT	1.72 (1.13-2.62)	0.85	1.38 (1.02-1.86)	0.002	2.57 (2.28-2.88)	0.12
H6: CAGTGIT	1.76 (1.21-2.56)	0.92	1.07 (0.82-1.38)	0.09	2.62 (2.36-2.89)	0.04
H7: CGGTGIT	1.63 (0.91-2.92)	0.74	0.91 (0.69-1.19)	0.61	2.58 (2.33-2.84)	0.07

‡log-transformed. §square root transformed.

Haplotype	HDL(mmol/l)‡	P value compared to AGGCGDT	LDL (mmol/L)§	P value compared to AGGCGDT	Ox-LDL/LDL (U/mmol)‡	P value compared to AGGCGDT
H1: AGGCGDT	0.62 (0.59-0.66)	-	1.22 (1.13-1.30)	-	9.58 (8.58-10.70)	-
H2: CGACDIT	0.62 (0.57-0.67)	0.90	1.53 (1.40-1.66)	<0.001	8.15 (6.79-9.79)	0.17
H3: CGGCGIG	0.66 (0.60-0.71)	0.33	1.44 (1.29-1.59)	0.02	8.48 (6.69-10.75)	0.37
H4: AGGCDIT	0.70 (0.62-0.79)	0.10	1.49 (1.28-1.71)	0.02	7.70 (5.81-10.21)	0.16
H5: AGGCGIT	0.56 (0.53-0.65)	0.19	1.45 (1.16-1.77)	0.14	9.26 (6.90-12.43)	0.83
H6: CAGTGIT	0.62 (0.54-0.70)	0.86	1.41 (1.15-1.70)	0.18	7.62 (5.90-9.83)	0.14
H7: CGGTGIT	0.70 (0.59-0.83)	0.23	1.36 (1.14-1.61)	0.26	6.50 (5.04-8.38)	0.01

‡log-transformed. §square root transformed.

Table 7.23 B: p values for other associations tested

Haplotype	P value for % sdLDL	P value UKPDS risk score	P value %TAOS
H1: AGGCGDT	-	-	-
H2: CGACDIT	0.93	0.32	0.38
H3: CGGCGIG	0.54	0.20	0.76
H4: AGGCDIT	0.39	0.98	0.94
H5: AGGCGIT	0.35	0.66	0.10
H6: CAGTGIT	0.94	0.02	0.46
H7: CGGTGIT	0.35	0.87	0.26

7.4.6 Evolutionary relationship of Haplotypes

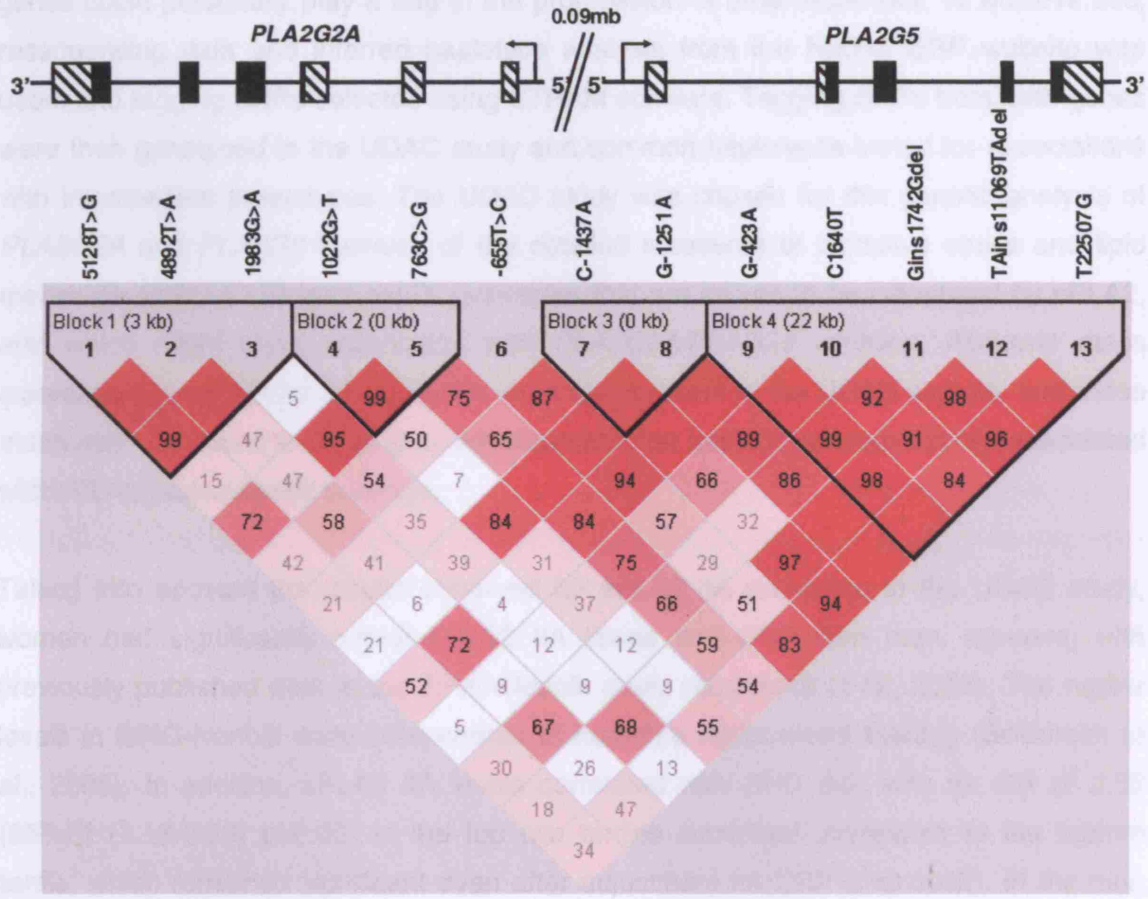
Constructing the evolutionary relationship of the inferred common haplotypes in *PLA2G5* proved to be inconclusive, with several combinational changes of tSNPs possible for each haplotype. As such, a graphical representation of this pattern proved unobtainable. However, it is interesting to note that all of the TG, LDL and cholesterol level raising haplotypes (H2, H3 and H4) shared the TAins11609 genotype compared to the 11609TAdel genotype in H1. Analysis of the TAins11609TAdel variant in single SNP analysis shows that the TAins11609 genotype is significantly associated with raised LDL and cholesterol levels (a borderline significant p=0.06 association with TG levels), which itself corresponds to the observed associations of these common haplotypes (Table 7.20).

7.5 Combined analysis of the *PLA2G2A* and *PLA2G5* haplotypes

7.5.1 LD pattern across the *PLA2G2A* and *PLA2G5* gene cluster

Figure 7.8 shows the combined LD pattern (D') as measured by the Haploview 3.2 program (Broad institute, USA). The Figure shows that LD across the two genes is not strong, with low LD between *PLA2G2A* and *PLA2G5* tSNPs. This suggests that possible recombination between the two genes may exist. However, no SNPs were genotyped in the 90 Kb of DNA between the two genes, potentially distorting the pattern seen here. Interestingly, the association of certain individual *PLA2G5* tSNPs with sPLA2 IIA mass levels (C-1437A, G-423A, Gins1742Gdel, and TAins11069TAdel in particular) could be the result of the strong LD that appears to exist between these SNPs and the T-655C and C763G *PLA2G2A* tSNPs (both of these SNPs showed strong associations with sPLA2 IIA levels, p<0.0001).

Fig 7.8: Map of the *PLA2G2A* and *PLA2G5* genes in their correct orientation. Haploview LD (D') of tSNPs is shown below. The darker boxes represent the stronger LD. The D' for any two SNPs is presented in the box representing their intersection. No number indicates a D' of 1.



7.5.2 Combined haplotype associations with intermediate phenotypes in UDACS

As discussed in the introduction of this chapter, *PLA2G2A* and *PLA2G5* genes have very similar potential roles in atherosclerosis and are located in close proximity to each other (within 90kb). Unfortunately, we were unable to further investigate haplotypes encompassing all 13 tSNPs in these genes (combined haplotype analysis of both genes) due to computational limitations regarding the large number of tSNP markers and potential inferred haplotypes. In addition, without the proper selection of tSNPs covering the entire region of both genes, including the chromosomal region between the *PLA2G2A* and *PLA2G5* genes, it was not possible to accurately infer haplotypes.

7.6 Discussion

The specific aim of this chapter was to investigate whether the *PLA2G2A* and *PLA2G5* genes could potentially play a role in the progression of atherosclerosis. To achieve this, resequencing data and inferred haplotype analysis from the NIEHS EGP website was used, and tagging SNPs selected using STRAM software. Tagging SNPs from both genes were then genotyped in the UDAC study and common haplotypes tested for associations with intermediate phenotypes. The UDAC study was chosen for this genetic analysis of *PLA2G2A* and *PLA2G5* because of the detailed measures of oxidative stress and lipid measures such as LDL and oxLDL; variables that are known to be influenced by sPLA2, and which might show association with *PLA2G2A/PLA2G5* variation. Accurate mass measurements of sPLA2 IIA by ELISA were also present in the UDAC sample, and these measures were used to investigate whether common haplotypes were directly associated with sPLA2 protein levels in serum.

Taking into account the results observed for sPLA2 IIA measures in the UDAC study, women had significantly higher sPLA2 IIA levels and CRP than men, agreeing with previously published data in the EPIC-Norfolk study (Boekholdt et al., 2005). The higher levels in EPIC-Norfolk were independent of hormone replacement therapy (Boekholdt et al., 2005). In addition, sPLA2 IIA levels correlated with CHD risk, with an OR of 2.50 (95%CI (1.13-5.53) p=0.02) in the top two tertiles combined, compared to the bottom tertile, which remained significant even after adjustment for CRP ($p<0.0002$). In the men the same trend was evident, but did not reach statistical significance. In the men sPLA2 IIA levels showed strong negative correlation with HDL cholesterol, seen previously in the EPIC-Norfolk study (Boekholdt et al., 2005), and with TAOS, a net measure of plasma oxidative stress, with higher total anti-oxidant status, equating to lower the oxidative stress.

As discussed in chapter 4 (section 4.4.4), TAOS is not a highly specific measure of plasma oxidative stress, but for a large number of samples it is a practical, inexpensive assay and there is evidence supporting the use of plasma TAOS as a marker of plasma oxidative stress (Stephens et al., 2004) with, for example, a strong correlation (r=-0.65) between plasma TAOS and the gold-standard measure of oxidative stress, esterified F_2-isoprostane levels (p=0.003)(Stephens et al., 2004). The secretory PLA2 IIA mass measures in UDACS were taken using a highly specific sPLA2 IIA commercially available ELISA kit

(Cayman Chemical company, Ann Arbor, MI), which has been shown by the manufacturer to not cross react with any other related sPLA2 enzymes, including the closely related sPLA2 V protein (www.caymaneurope.com). Unfortunately, no sPLA2 IIA activity assay is currently commercially available. The inverse relationship between sPLA2 IIA and TAOS is supported by a recent study demonstrating that macrophage-specific over-expression of sPLA2 IIA, after bone marrow transplantation from sPLA2 IIA transgenic mice, accelerated atherogenesis in *ldlr-/-* mice, with an increase in oxidative stress as measured by F2-isoprostanes (Tietge et al., 2005). The results observed in UDACS add weight to the concept that the increased oxidative stress associated with high sPLA2 IIA levels provides an additional mechanism for the pro-atherogenic role of sPLA2 IIA.

***PLA2G2A* gene associations**

The second aim of this chapter was to investigate variation in the *PLA2G2A* gene and its association with sPLA2 IIA levels and intermediate phenotypes. In the UDAC study, an association was found between sPLA2 IIA mass levels and CHD risk, in agreement with previously published data (Boekholdt et al., 2005; Hurt-Camejo et al., 2001; Kugiyama et al., 1999; Liu et al., 2003). Previous *in vivo* investigations have revealed that sPLA2 IIA localises specifically to atherosclerotic plaques and is often found in the intimal space of arteries (Romano et al., 1998; Menschikowski et al., 1995a; Hurt-Camejo et al., 1997). In addition, transgenic mouse studies have also demonstrated that those mice with the human *PLA2G2A* transgene are at a greater risk of CHD (Ivandic et al., 1999; Leitinger et al., 1999).

Despite the mounting body of evidence supporting a link between sPLA2 IIA and atherosclerosis, causality of this enzyme has yet to be firmly established. In traditional epidemiological studies the association between phenotype and disease is often biased by confounding and reverse causation (Smith and Ebrahim, 2004; Casas et al., 2005). Secretory PLA2 IIA itself is regarded as an inflammatory enzyme under tight transcriptional control from other inflammatory stimuli (Andreani et al., 2000; Fan et al., 1997; Paradon et al., 1998; Peilot et al., 2000); it is therefore possible that the association of sPLA2 IIA with CHD may represent a simple marking of the inflammatory state associated with atherosclerosis. Although statistical adjustment makes some allowance for this type of confounding, residual confounding is a concern, because in any study not all confounders are known or measured, and those which are measured are measured with error, making

complete adjustment difficult. However, as discussed in chapter 4, genotype is not subject to confounding since it is determined at conception by the random inheritance of one of each parental allele (Minelli et al., 2004; Thomas and Conti, 2004). Common genetic variants in the secretory PLA2 genes are therefore potentially useful tools for overcoming this confounding effect.

In order to test whether sPLA2 IIA plays a causative role in atherosclerosis, a tagging SNP approach was used to investigate whether genetic variation in the *PLA2G2A* gene was associated with sPLA2 IIA levels and also markers of atherosclerosis in an 'at risk' group of T2DM individuals. From the NIEHS EGP website it was possible to obtain re-sequencing data of the *PLA2G2A* gene region in 90 individuals of mixed race, along with inferred haplotypes from PHASE. Genotyping of the UDAC study using the selected tSNPs revealed weak LD across the gene region, although a distinct LD block did appear 3' of exon 3. Using THESIAS it was then possible to identify 6 common haplotypes. Unfortunately, the inferred common haplotypes from the PDR 90 population [EGP resource (Livingston et al., 2004)] did not match those found in the UDAC study. It is important to consider that the process of haplotype and tSNP analysis is dependent on consistent LD pattern within the cohort under investigation (Lin et al., 2002). As such, the assumptions made about LD can be compromised by population heterogeneity in the tested sample (Suh and Vijg, 2005; Salisbury et al., 2003; Schneider et al., 2003). The PDR 90 cohort consists of several different ethnic backgrounds (Livingston et al., 2004), each with a distinct ancestral genetic pool, LD pattern and haplotype structure. Combining different geographical and ethnic groups may have artificially interfered with the inferred haplotypes detected by the PHASE program. This in turn may explain why the tSNPs generated significantly different haplotypes in UDACS, a study that was carefully selected to only include a more homogeneous Caucasian population.

Overall, the 6 common haplotypes identified in UDACS were associated with highly significant effects on sPLA2 IIA levels, $p<0.00001$, confirming that variation in *PLA2G2A* was contributing to sPLA2 variance. The common *PLA2G2A* haplotype, H1, was associated with 53% higher sPLA2 IIA levels compared with the pooled other haplotypes. Secretory PLA2 IIA is thought to be active in hydrolysing the phospholipids present in lipoprotein particles, with the generation of lyso-phospholipids and FFA (Hurt-Camejo et al., 2001; Hurt-Camejo and Camejo, 1997; Oestvang et al., 2004). As a consequence

sPLA2 IIA can have multiple effects on LDL particle size, density and susceptibility to lipid peroxidation (Leitinger et al., 1999; Hurt-Camejo and Camejo, 1997; Sartipy et al., 1999; Hakala et al., 1999; Gorshkova et al., 1996; Kleinman et al., 1988; Menschikowski et al., 1995b). This raised the possibility that lipid measures of small dense LDL, oxLDL/LDL as well as measures corresponding to oxidant status (TAOS) could also be associated with the observed haplotypes in UDACS. Disappointingly, none of these intermediate traits were significantly associated with variation in the *PLA2G2A* gene. The reasons for this discrepancy are not clear, although the contribution of variation in the *PLA2G2A* gene to the variance in sPLA2 IIA levels was comparably small. It is conceivable that the subtle effects of the haplotypes in this study were not large enough to detect an association in these global markers of disease. This could also explain why the correlation of TAOS with sPLA2 IIA levels was not replicated in the haplotype analysis.

Unfortunately UDACS was an inadequately powered study to detect an association between genotype and CHD risk and was therefore unable to provide definitive evidence of sPLA2 IIA causality. A test of causality using mendelian randomisation (Thomas and Conti, 2004; Minelli et al., 2004) could be achieved by studying the relationship between CHD risk and a genetic determinant (in this case the *PLA2G2A* haplotypes) of an intermediate phenotype (sPLA2 IIA levels). If a causal relationship between *PLA2G2A* haplotypes and sPLA2 IIA mass levels were clearly established, an association between *PLA2G2A* haplotypes and CHD risk would provide indirect evidence for the causality of the association between sPLA2 IIA and CHD. This chapter has essentially addressed the first part of this test, by identifying a strong relationship between tSNP determined haplotypes and sPLA2 IIA mass levels. However, there is still a need for large prospective and case-control studies to resolve the relationship between these common haplotypes and CHD risk.

***PLA2G5* gene associations**

In contrast to work carried out on the *PLA2G2A* gene and its related protein, limited research has focused on the contribution of a structurally similar sPLA2 enzyme, group V, in the progression of atherosclerosis. As with sPLA2 IIA, the group V enzyme was originally thought to play a role as an anti-bactericidal agent specifically acting against the phospholipid membranes of gram-positive bacteria (Koduri et al., 2002). Recent work has suggested that sPLA2 V may also be a potent pro-atherogenic enzyme. *In vitro* work has

demonstrated that sPLA2 V is 20 times more active than sPLA2 IIA in its ability to hydrolyse phospholipids (Pruzanski et al., 2005), with a suggestion that sPLA2 V binds and hydrolyses PC phospholipid membranes twice as efficiently as the sPLA2 IIA enzyme (Han et al., 1998). Recently, sPLA2 V has also been found to act in a similarily pro-atherogenic way to sPLA2 IIA, with evidence that sPLA2 V promotes foam cell formation through the aggregation and fusion of lipoprotein particles (Boyanovsky et al., 2005; Murakami and Kudo, 2003; Wooton-Kee et al., 2004). Unfortunately, no commercially available ELISA kit exists to measure mass levels of sPLA2 V in plasma, making it difficult to determine whether enzyme levels are directly associated with CHD in human populations.

The third aim of this chapter was to investigate genetic diversity in the *PLA2G5* gene and relate this to potential markers of disease. The location of the *PLA2G5* gene is very close to that of *PLA2G2A*, but Selection of tSNPs in the *PLA2G5* gene region and subsequent genotyping in UDACS revealed a strong pattern of LD across the gene, in stark contrast to the more sporadic LD found in the *PLA2G2A* gene. This may explain why the tSNP approach used for the *PLA2G2A* gene region detected 6 common haplotypes that only accounted for 68% of the genetic variation within the gene, while the seven common haplotypes in the *PLA2G5* gene accounted for 92% of genetic variation.

Since no measures of sPLA2 V mass were available, intermediate phenotypes related to the oxidation of LDL and alterations in particle size were investigated alongside measurements of TAOS. In haplotype analysis it was shown that haplotypes H2, H3, H4 and H5 were associated with significantly higher lipid measures, with the H2 haplotype in particular showing a 29.8% raised TG level, 13.8% higher cholesterol level, and 25.4% LDL level when compared to H1. These highly significant associations are hard to interpret because of the lack of group V mass measures which would be able to determine whether the raising effects seen in these haplotypes are a consequence of an alteration in sPLA2 V level in the bloodstream. However, a potential mechanism has been suggested whereby sPLA2 IIA modification of lipoproteins leads to the retention of LDL particles within the intima and the uptake of entire particles by the surface transfer process (in the arterial wall this would lead to macrophage foam cell formation) (Jaross et al., 2002). This would then result in a faster clearance of cholesterol from the blood stream (Jaross et al., 2002). The

process could also apply to the sPLA2 V enzyme, explaining the alterations in cholesterol, TG, and LDL seen in UDACS.

In contrast to the positive associations relating to lipid levels, only the comparatively rare haplotype H7 showed a significant association with oxLDL/LDL levels, and there were no further associations with sdLDL, TAOS, or UKPDS risk score. As with *PLA2G2A*, the reason for this lack of association is not entirely clear, and may relate to the sensitivity of these assays to detect subtle differences in lipid peroxidation, oxidant status and CHD risk. In addition, plasma levels of oxLDL/LDL and sdLDL may not entirely reflect the effect of sPLA2 enzymes in the intima of arteries. Clearly, there is the need to develop an ELISA assay that is specific for this enzyme. This would then enable large mendelian randomised approaches to determine the causality of this enzyme in a similar way to that of sPLA2 IIA.

Combined analysis

Both the *PLA2G2A* and *PLA2G5* genes exhibit very similar pro-atherogenic properties. Single SNP analysis of the *PLA2G5* gene showed that four of the selected 7 tSNPs in the *PLA2G5* gene (C-1437A, G-423A, Gins1742Gdel, and TAins11069TAdel) were associated with sPLA2 IIA measures. Since the IIA ELISA assay shows no cross-reactivity with the group V protein (according to the manufacturers manual), these associations may be the result of LD existing between tSNPs in both genes. Indeed, when the D' of all the tSNPs from both genes were compared (Fig. 7.8), the four *PLA2G5* tSNPs showing associations with mass levels were found to be in LD with tSNPs in the *PLA2G2A* gene. All but one (T4892A) of the tSNPs in the *PLA2G2A* gene showed strong associations with sPLA2 IIA mass levels, therefore it is possible that the effect seen in the *PLA2G5* tSNPs was a reflection of this. It would have been potentially worthwhile to investigate combined genetic variation in both the *PLA2G2A* and *PLA2G5* region; however, problems arose when trying to determine the most common inferred haplotypes using all 13 tSNPs. Firstly, the intergenic region between the two genes was not genotyped for any variation, preventing the determination of an accurate haplotype block structure across the whole region. There was also the observation in Figure 7.8 that LD drops off between the two genes. As a consequence, when THESIAS was used to determine the most commonly occurring haplotypes, a large number of rare haplotypes were produced with very few common haplotypes observed. This underpowered the study so that it was very difficult to analyse any associations. In some ways this shows the difficulties of haplotype analysis across

genetic regions of low LD: the lower the LD pattern, the larger the sample number needed to investigate haplotype associations (Seltman et al., 2003).

Finding the functional SNP

Haplotype analysis is an effective method by which to determine if candidate genes or regions containing candidate genes are associated with disease. However, haplotype analysis represents a 'first screening' effort, irrespective of the truly functional variant (Johnson et al., 2001; Suh and Vijg, 2005). In order to validate the associations found, and further understand the physiological role of a particular gene, screening for the functional SNP may be necessary (Suh and Vijg, 2005). In the case of *PLA2G2A* this was attempted by the use of a cladogram. Cladograms are the graphical representation of the evolutionary relationship of haplotypes and can be used to determine the chronological development of these haplotypes (Seltman et al., 2003). In the analysis of the *PLA2G2A* gene a cladogram was used to see if one particular tSNP explains the differences seen between haplotypes. In the case of *PLA2G2A*, the cladogram showed that no single SNP was found to be determining the changes in sPLA2 IIA mass levels seen between haplotypes. Unfortunately, the use of cladograms proved unsuitable for determining a tSNP responsible for the associations seen in the *PLA2G5* analysis. However, by looking at the common haplotypes associated with changes in lipid measures, it becomes clear that the TAins11069TAdel SNP is common to those lipid raising haplotypes (the individual SNP analysis of TAins11609TAdel also shows strong associations with these traits). This may present a first step towards determining the functional variant; the TAins11069TAdel variant may not be functional itself, but other SNPs in LD with this variant may explain the effects seen (Suh and Vijg, 2005). Preliminary searching of all the genotyped SNPs (from the PDR 90 population) in strong LD with this variant has not revealed any SNP with a potentially functional role.

In conclusion

The lack of epidemiological data regarding variation in the *PLA2G2A* and *PLA2G5* genes means that single SNP analysis was not a suitable technique by which to help clarify the causality of these genes regarding atherosclerosis. By using tSNP analysis, it was possible to investigate associations since these methods take into account most of the genetic variation present within the gene of interest. The associations found here regarding lipid measures and sPLA2 IIA mass levels need to be investigated further and

incorporated with studies examining CHD risk. Certain limitations to this method still exist, specifically regarding patterns of LD. There is also the question of eventually determining the functional SNP. However, haplotype analysis does represent an effective primary approach for identifying genes involved in complex phenotypes such as atherosclerosis (Goldstein, 2001; Suh and Vijg, 2005).

7.7 Summary of Results

i) sPLA2 IIA mass levels were found to be significantly elevated in women compared to men. In men sPLA2 IIA levels were negatively correlated with %TAOS and HDL. IIA mass levels were also positively correlated with CRP. In women, sPLA2 IIA levels correlated with the proportion of CHD events.

ii) Using a tSNP approach it was possible to identify common inferred haplotypes of the *PLA2G2A* and *PLA2G5* genes in the UDAC study.

iii) *PLA2G2A* haplotypes were significantly associated with differences in sPLA2 IIA mass levels, but not intermediate phenotypes relating to lipid and inflammatory markers.

iv) *PLA2G5* haplotypes were associated with TG, Cholesterol, LDL and oxLDL/LDL levels in the UDAC study. Unfortunately, sPLA2 V measures are currently unavailable.

v) It was not possible using this tSNP approach to identify a truly functional variant, however, the haplotype approach appeared to be an effective method to test causality of specific genes in relation to the progression of atherosclerosis.

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

8.1 Thesis Conclusion

The Phospholipase A2 enzyme family consists of a group of enzymes with the shared ability to hydrolyse the sn-2 position of phospholipids, resulting in the production of free fatty acids and lysophospholipids (Six and Dennis, 2000). The work presented in this thesis has concentrated on three members of this family which are closely linked through their specific activities to the regulation of potent phospholipid mediators and the oxidative modification of LDL particles. As such, these enzymes represent important modulators of both inflammation and lipid metabolism; processes regarded as critical in the progression of atherosclerosis.

The Lp-PLA2 enzyme has been previously identified as a marker of CHD risk in several prospective and case-control epidemiological studies [reviewed in (Sudhir, 2005)]. However, this enzyme remains a controversial candidate for cardiovascular disease since it shows both pro- and anti- atherogenic properties. Lp-PLA2 is able to hydrolyse PAF and PAF-like substances which are known to be strong pro-inflammatory molecules (Tjoelker and Stafforini, 2000). On the other hand, Lp-PLA2 has the ability to hydrolyse oxidised PC resulting in the generation of pro-inflammatory Lyso-PC and oxidised free fatty acids (Macphee et al., 1999). In order to determine which of the pro- or anti- atherogenic properties of Lp-PLA2 were predominant; my thesis concentrated on investigating the relationship of genetic variation in the *PLA2G7* gene with enzyme activity, markers of atherosclerosis and CHD risk. In particular, a direct candidate SNP approach was used by analysing the putatively functional *PLA2G7* A379V variant (Kruse et al., 2000). In addition, the promoter region of the *PLA2G7* gene was screened for novel polymorphisms in an effort to detect new functional variants for investigation. A final aim was to determine whether statins, which are known to have effects on inflammatory responses, also directly regulate *PLA2G7* gene expression. These epidemiological and functional analyses would allow the causality of the Lp-PLA2 enzyme to be thoroughly investigated.

The second objective of this thesis was to investigate the contribution of the secretory PLA2 enzyme cluster on chromosome 1. The *PLA2G2A* and *PLA2G5* genes have been identified as potential modulators of inflammation through their ability to breakdown phospholipid membranes, with the subsequent production of pro-inflammatory mediators (Hurt-Camejo and Camejo, 1997; Hurt-Camejo et al., 2001; Jaross et al., 2002). Unlike the

Lp-PLA2 enzyme, these sPLA2 enzymes play a limited role in the degradation of PAF and may therefore have entirely pro-atherogenic properties. In contrast to the direct candidate SNP approach conducted in the Lp-PLA2 analysis, I used a tagging SNP approach that tried to ascertain whether genetic variation in these two genes were related to markers of CHD, and also mass levels of sPLA2 IIA. This would hopefully help determine the functional relationship of these enzymes with atherosclerosis, and provide a useful comparison of two population based genetic approaches for investigating the causal relationship of certain enzymes with complex disorders such as atherosclerosis.

8.1.1 The relationship of *PLA2G7* genotype and Lp-PLA2 activity with CHD risk

The relationship of Lp-PLA2 activity to CHD risk

Previously, several published studies have shown robust associations of Lp-PLA2 activity and/or mass with markers of CHD [reviewed in (Sudhir, 2005)]. The aim of this thesis was to add to this data by providing additional information on genotype (in particular the A379V variant), Lp-PLA2 activity, intermediate phenotypes and CHD risk in large, well powered studies. Taking into account the Lp-PLA2 activity data, conclusions from the EPIC-Norfolk study suggested a strong positive association of Lp-PLA2 activity with CHD risk ($p<0.0001$). Unfortunately, this association no longer remained statistically significant after adjusting for cholesterol ($p=0.45$). The loss of significance in this case must be considered in two ways: firstly from a diagnostic assay point of view, and secondly from the investigation of causality being attempted in this thesis. The failure to find a robust association of activity with CHD risk in EPIC-Norfolk after adjustment for cholesterol suggests that clinically measuring Lp-PLA2 activity will not provide additional information over and above measurement of classical risk factors such as cholesterol. On the other hand, adjustment for cholesterol when investigating the causal relationship of the Lp-PLA2 enzyme to CHD risk may not be suitable because of the association of the enzyme with LDL in plasma and its lipoprotein-independent production by haemopoietic cells. Interestingly, other studies investigated in this thesis also showed similar trends regarding Lp-PLA2 activity and risk of CHD. The NPHS II study was less well powered than the EPIC-Norfolk study to pick up an association, but a positive trend was still apparent with those individuals with higher Lp-PLA2 activity being at a higher risk of CHD. Further analysis in the diabetic cohort of the UDAC study showed a significant positive association of Lp-PLA2 activity with the UKPDS risk score ($p=0.006$), and Lp-PLA2 activity was also found to be 7.9% higher in those diabetic individuals with features of the MS ($p=0.02$). This thesis represents one of the first studies to investigate the association of Lp-PLA2 activity with the MS, and shows that Lp-PLA2 is a good predictor in those individuals at higher risk of developing CHD.

Overall, this thesis has demonstrated that higher Lp-PLA2 activity is associated with higher CHD risk. The failure to find a statistically robust association across a range of studies has more to do with the different study designs used in this thesis. The lack of a significant association in the NPHS II study could well have been caused by a lack of power in the

study, while lower Lp-PLA2 activity measures in those individuals with CHD (compared to those without) in the UDAC study could have been confounded by intensive medication. However, the failure to confirm an independent association of Lp-PLA2 activity with CHD risk does not allow the full endorsement of Lp-PLA2 activity as a clinical marker when considering the results from this thesis as a whole.

Association of Lp-PLA2 activity with other intermediate phenotypes

Apart from investigating the causal relationship of the Lp-PLA2 enzyme with CHD, this thesis aimed to investigate the association of Lp-PLA2 activity with intermediate phenotypes known to be important in the progression of atherosclerotic disease. In the studies where Lp-PLA2 activity measures were available (EPIC, NPHS II and UDACS), there were significant associations of Lp-PLA2 activity with various lipid markers such as cholesterol, HDL, LDL and triglycerides. These observations agreed with previously published data (Sudhir, 2005), and reflect the fact that the Lp-PLA2 enzyme is known to be bound to LDL and HDL in circulation (Noto et al., 2003; Kujiraoka et al., 2003). However, the significant inverse relationship of oxLDL/LDL levels and Lp-PLA2 activity found in UDACS ($p=0.03$) provided novel information about the relationship of Lp-PLA2 enzyme and lipoproteins; and could explain both the pro- and anti- atherogenic properties of this enzyme. The negative association of Lp-PLA2 enzyme with oxLDL/LDL suggests at one level that the Lp-PLA2 enzyme is protective in terms of LDL oxidation, in agreement with previously published data (Lee et al., 1999; Noto et al., 2003). Conversely, evidence has shown that the products of Lp-PLA2 mediated hydrolysis of oxidised PC results in the generation of lyso-PC and free fatty acids with resultant pro-atherogenic properties (Carpenter et al., 2001; Macphee et al., 1999; Tew et al., 1996). Although the Lp-PLA2 enzyme may be protecting LDL particles from oxidation, it could be releasing potent pro-atherogenic mediators such as lyso-PC and oxidised free fatty acids at the same time. Unfortunately, without complex phospholipid biochemical analysis no firm conclusions could be made about this interesting association. However, the lack of a significant association between TAOS and Lp-PLA2 activity in UDACS ($p=0.19$) would suggest that whatever the role of Lp-PLA2 in LDL oxidation, the effects do not impact on global oxidative stress.

In conclusion this thesis has confirmed previously reported associations of lipid markers with Lp-PLA2 enzyme activity. In addition, it has been shown that there is a highly

significant interaction of the Lp-PLA2 enzyme with oxidised LDL that warrants further investigation using *in vitro* and *in vivo* models.

A379V genotype associations with Lp-PLA2 activity, intermediate phenotypes and CHD risk

The conclusions relating Lp-PLA2 activity to markers of atherosclerosis and CHD risk are still susceptible to confounding factors which prevent the clarification of a pro- or anti-atherogenic role. As a result of this confounding, the association of higher Lp-PLA2 activity with CHD risk could be explained in one of three ways: firstly, the enzyme could simply be marking the disease state with no causal link with CHD disease (confounding). Secondly, the higher levels of Lp-PLA2 activity in those individuals with CHD could represent a physiological adaptive response to the diseased state (reverse-causation). Or thirdly, there could indeed be a causal relationship of the enzyme with CHD disease.

Mendel's second law assumes that alleles are assigned randomly at birth, a concept termed Mendelian randomisation (Minelli et al., 2004; Thomas and Conti, 2004). A test of causality free from confounding could be achieved by studying the relationship between CHD risk and a genetic determinant of an intermediate phenotype. Previous studies have investigated the relationship of Lp-PLA2 mass and activity measures with CHD risk, while the AtheroGene and HIFMECH studies were the first to show a significant association of the putatively functional *PLA2G7* A379V variant with CHD risk (Abuzeid et al., 2003; Ninio et al., 2004). However, a central aim of this thesis was to determine a robust relationship between genetic variation in the *PLA2G7* gene, enzyme activity and CHD risk: if a causal relationship between *PLA2G7* genotype and activity were clearly established, a significant association between *PLA2G7* genotype and CHD risk would provide indirect evidence for the causality of the association between Lp-PLA2 activity and CHD risk. Genotyping of the *PLA2G7* A379V variant in several large prospective (NPHSII), case-control (EPIC-Norfolk) and cross-sectional (UDACS) studies would hopefully confirm the associations seen initially in the HIFMECH and AtheroGene studies, while also providing comprehensive Lp-PLA2 activity data and enabling a better understanding of the causal relationship of this enzyme with CHD.

Previous *in vitro* data has shown the 379V allele to be associated with a lower PAF substrate affinity (Kruse et al., 2000). However in contradiction to this, and in agreement with the recently published data from the AtheroGene study (Ninio et al., 2004), results presented in the NPHS II and EPIC-Norfolk analysis (UDACS failed to show a significant association) suggested that the 379V allele was associated with a subtly higher Lp-PLA2 activity [NPHS II p=0.11 (p=0.05 trend), and EPIC-Norfolk p=0.03]. In addition, analysis of a smaller subset of the NPHS II study also showed that the Lp-PLA2 specific activity of 379V homozygous individuals was significantly higher compared to A379 homozygous men (p=0.001, p=0.14 after adjustment). The measurement of specific activity takes into account plasma differences in Lp-PLA2 mass and more accurately quantifies differences in Lp-PLA2 activity. The data presented in this thesis represents the first investigation of Lp-PLA2 specific activity in relation to *PLA2G7* A379V genotype, and suggests that this measurement may provide additional information in epidemiological studies over and above individually measuring Lp-PLA2 mass or activity. Apart from the significant relationship of A379V genotype with Lp-PLA2 enzyme activity, analysis of the NPHS II study also showed that there was a significant association of higher cholesterol levels with the 379V allele (p=0.0006). EPIC failed to confirm this observation, but did show that those homozygous for the 379V allele had lower HDL levels in a recessive model (p=0.03). If the 379V allele is indeed associated with higher activity, then the association of genotype with cholesterol and HDL correctly reflects the previously observed relationship of Lp-PLA2 activity with lipid markers. The relationship of A379V genotype with both Lp-PLA2 activity and lipid markers would suggest that at some level the A379V variant is having a relevant functional effect.

When considering the association of A379V genotype with CHD risk, the HIFMECH and AtheroGene studies have shown an independent association of the 379V allele with lower risk of CHD (Abuzeid et al., 2003; Ninio et al., 2004). If the 379V allele was associated with higher activity but also lower CHD risk, then one could infer that the Lp-PLA2 enzyme may be anti-atherogenic. However, in this thesis the NPHS II study failed to find a significant association of the 379V allele with lower CHD risk (p>0.63 in all models), and in the EPIC-Norfolk study those individuals homozygous for the 379V allele were associated with a borderline significant *higher* CHD risk (this association was only present in the recessive model, p>0.06). Interestingly, if the borderline significant effect seen in the EPIC-Norfolk study was due to alterations in Lp-PLA2 enzyme activity, then over-adjustment of the

multivariate model by including Lp-PLA2 activity should have removed this association. However, this over adjustment of the model did not significantly alter the observation of genotype with CHD risk, implying that the relationship of A379V genotype with risk was independent of the Lp-PLA2 plasma activity measures in this particular study.

In conclusion, this thesis has shown that there is a weak but significant association of the 379V allele with higher plasma Lp-PLA2 activity, contradicting previous *in vitro* observations (Kruse et al., 2000). Because of this, the functional relevance of the A379V variant needs to be analysed further since the change in activity associated with this variant is small and unlikely to fully explain the previously reported relationship of A379V genotype with CHD risk (Ninio et al., 2004). In addition to these observations, the association of A379V genotype with CHD risk seen in the HIFMECH and AtheroGene studies was not confirmed in the larger prospective NPHS II and EPIC-Norfolk nested case-control studies. The failure to confirm the association of A379V genotype with CHD risk seen in smaller published studies raises questions about whether the Lp-PLA2 enzyme is causally related to the progression of atherosclerosis based on the data presented here. The contradictory data regarding A379V genotype and Lp-PLA2 activity also questions the validity of using this variant as a marker of enzyme function in association studies. However, the large body of genetic evidence now collected from this thesis and other published studies should enable a comprehensive meta-analysis of the available genetic data, from which an accurate assessment of causality could be inferred by using the concept of Mendelian randomisation.

The association of A379V genotype with body composition

Apart from efforts to determine the triangular relationship of *PLA2G7* A379V genotype, Lp-PLA2 activity and CHD risk, this thesis aimed to examine whether the Lp-PLA2 enzyme was involved with other important physiological processes that may relate to the progression of atherosclerosis. Through the remodelling pathway of PAF, Lp-PLA2 has the ability to indirectly modulate several different cell signalling molecules thought to be involved in adipose tissue and lean mass regulation, such as AA and LPA (Balsinde et al., 2002; Moolenaar et al., 2004; Ninio, 2005). The BH2 study offered the opportunity to test the association of the A379V variant with changes in adipose tissue mass and lean mass caused by 10 weeks of intensive army basic training. Uniquely, this study contained an accurate quantitative assessment of human adipose and lean tissue mass through the use

of whole body MRI scans. Although there were no significant differences in BMI, weight, adipose tissue and lean mass by A379V genotype at baseline; there were significant differences in percentage change of lean mass and adipose tissue mass over the 10 week training period. The 379V allele was found to be associated with a significant increase in lean mass ($p=0.02$), matched by a similar lowering in percentage adipose tissue mass ($p=0.01$) over the 10 week training period. The significant linear trend with AV recruits exhibiting intermediate values, suggests that even though the numbers of 379V homozygotes were small ($n=7$), the conclusions were still valid. The design of shared environment, identical training, and accurate phenotypic measurement at baseline and follow-up also increased the power of the study considerably.

The data from the BH2 study is the first to investigate the indirect (through the use the A379V variant) relationship of the Lp-PLA2 enzyme with changes in body composition, although it was not possible to precisely determine the functional relationship of Lp-PLA2 enzyme with body composition in this study due to a lack of Lp-PLA2 activity measures. Clearly, there is a need for this association to be further investigated in a larger and more powerful study that also incorporates comprehensive plasma and serum analysis of Lp-PLA2 activity, AA and LPA levels. This in turn may help explain the mechanism behind the initial observations seen in the BH2 study. However, this study does demonstrate that the relationship of Lp-PLA2 enzyme with atherosclerosis may be more complicated than originally contemplated, with PLA2 enzymes potentially modulating other processes involved in CHD, such as adipogenesis.

8.1.2 Identification of the novel G-1230A SNP and subsequent functional studies

Chapters 3, 4 and 5 of this thesis also described the detection and subsequent analysis of a novel *PLA2G7* promoter variant. Several variants have already been reported in the *PLA2G7* gene, with haplotype analysis demonstrating that only the A379V variant showed an independent effect on CHD risk and enzyme activity (Ninio et al., 2004). Using SSCP and heteroduplex analysis it was possible to identify a novel G to A variant 1230bp 5' of the previously described *PLA2G7* transcription start site (Cao et al., 1998), and subsequent genotyping revealed the SNP to be in strong LD with the A379V variant (D'= -0.90. p=<0.0005). The association of this novel SNP with intermediate phenotypes, Lp-PLA2 activity and CHD risk were examined in the UDAC, BH2, and HIFMECH studies. In the HIFMECH study there appeared to be no association of the G-1230A genotype with CHD risk in any of the four individual centres and in combined analysis (p>0.22 in combined centre analysis). Additional analysis in the UDAC study showed no association of G-1230A genotype with either Lp-PLA2 activity (p=0.77) or other intermediate phenotypes (combined genotype analysis of the G-1230A and A379V variants failed to show any additive effects for those intermediate phenotypes tested). There was also no association found between the G-1230A SNP and changes in body composition in the BH2 study. Despite the failure to identify any significant associations in this thesis relating to the G-1230A SNP, a functional role could not be entirely ruled out without using a functional assay. In parallel with the association study analysis, a dual luciferase reporter assay was developed to investigate if the G-1230A SNP influenced *PLA2G7* promoter function. In Huh-7 cells successfully transfected with the pGL3-basic vector containing the relevant *PLA2G7* promoter constructs, there were no significant differences in luciferase activity between the G-1230 and -1230A constructs, suggesting that the variant may not be functional. Subsequent bioinformatics analysis suggested the G-1230A SNP did not disrupt any putative transcription factor binding sites. Further work is currently underway in an attempt to see whether the previously identified T-403C and C-209G variants found by Ninio *et al.* (Ninio et al., 2004) also have a functional effect, or whether all three SNPs have an effect in specific haplotype combinations. With the A379V variant showing inconsistent results in this thesis, especially regarding its association with CHD risk, it may prove beneficial to determine other functional variants in the *PLA2G7* gene. The use of the gene reporter assay also enables a comparably low cost and rapid functional assay for investigating promoter and 3' UTR variants.

8.1.3 *PLA2G7* gene expression and Statins

Analysis of the UDAC study in this thesis showed that Statin therapy was associated with a significant reduction in Lp-PLA2 activity (p=0.04, p=0.56 after adjustment for LDL). Previously published epidemiological data is in agreement with this result and has suggested that much of the reduction in Lp-PLA2 activity/mass can be accounted for by an associated lowering of LDL levels (Schaefer et al., 2005; Winkler et al., 2004). However, there is the possibility that statins may exhibit a direct effect on *PLA2G7* expression. Statins are known to down-regulate several inflammatory cytokines responsible for the transcriptional regulation of a number of inflammatory genes including *PLA2G7* (Cao et al., 1998; Davignon, 2004; Rezaie-Majd et al., 2002; Schonbeck and Libby, 2004; Schwartz and Olsson, 2005). In addition, the *PLA2G7* promoter region contains several binding sites for the RORalpha transcription factor, which is modulated by intracellular cholesterol levels (themselves affected by statins)(Kallen et al., 2002; Willson, 2002).

With that in mind, 9 healthy middle aged men from the NPHS II study who were not on statin medication were recruited and bled; monocytes cultured (differentiated into macrophages); and subsequently exposed to simvastatic acid. Using a two step RT-PCR Taqman based system it was possible to determine differences in expression of the *PLA2G7* gene caused by statin treatment when compared to control samples. Treatment with 10μM and 25μM statin led to an overall 17.6% and 27.2% reduction respectively in *PLA2G7* gene expression after 48 hours (p=0.01 and p=0.003 respectively). The results presented here are in direct contrast to those of Tsimihodimos *et al.*(Tsimihodimos et al., 2002), who found that Lp-PLA2 activity was elevated *in vitro* upon Atorvastatin treatment of human macrophages, while mRNA levels stayed constant. However, this study used β-actin as an endogenous control, which was shown in my analysis to be unsuitable as a stably expressed control gene in human derived monocyte-macrophages.

In conclusion, this thesis has shown for the first time that there is an LDL-independent lowering effect of Simvastatin on *PLA2G7* gene expression, although there is clearly a need for further work to clarify the nature of this relationship and whether this represents another anti-inflammatory pleiotropic aspect of statins *in vivo*.

8.1.4 Haplotype analysis of the *PLA2G2A* and *PLA2G5* genes

Apart from characterising further the Lp-PLA2 enzyme and its role in atherosclerosis, a second objective of this thesis was to initiate a genetic investigation of the *PLA2G2A* and *PLA2G5* genes. Both of these secretory PLA2 enzymes have been identified as potential candidates in the progression of atherosclerosis, since they exhibit pro-atherogenic properties both in the circulation and the arterial wall (Hurt-Camejo et al., 2001).

When considering sPLA2 IIA mass levels in UDACS, women had significantly higher sPLA2 IIA levels than men ($p<0.0001$), agreeing with previously published data in the EPIC study (Boekholdt et al., 2005). Secretory PLA2 IIA mass in women was associated with CHD risk, with an OR of 2.50 (95%CI (1.13-5.53) $p=0.02$) in the top two tertiles combined, compared to the bottom tertile, which remained significant even after adjustment for CRP ($p=0.002$) (in the men the same trend was evident but did not reach statistical significance, $p>0.21$). In the men, sPLA2 IIA levels showed strong negative correlation with HDL cholesterol ($p=0.006$ after adjustment), seen previously in the EPIC study (Boekholdt et al., 2005), and with TAOS ($p=0.003$ after adjustment), a net measure of plasma oxidative stress, with higher total anti-oxidant status, equating to lower the oxidative stress. The inverse relationship between sPLA2 IIA and TAOS is supported by recent data showing that macrophage-specific over-expression of sPLA2 IIA accelerated atherogenesis in *ldlr-/-* mice, with an increase in oxidative stress as measured by F2-isoprostanes (Tietge et al., 2005). The results observed in UDACS add weight to the concept that the increased oxidative stress associated with high sPLA2 IIA levels provides an additional mechanism for the pro-atherogenic role of sPLA2 IIA.

However, despite robust associations of sPLA2 (namely sPLA2 IIA) with CHD and plausible biological mechanisms to support this link, observational studies to date are again susceptible to confounding. In contrast to the direct candidate SNP approach used in the epidemiological analysis of the Lp-PLA2 enzyme, I decided to use an indirect 'tagging SNP' approach that uses a minimal number of variants in order to define the common haplotypes within the genetic region of interest. This approach while not ascertaining the functional variant directly, does offer an effective method by which to determine if genetic variation within a gene is having an effect on either an intermediate phenotype or CHD risk. In this case, neither the *PLA2G2A* or *PLA2G5* genes had been

well characterised with regards to putatively functional variants, making this approach a more appropriate method of investigating the causal relationship of the two sPLA2 enzymes with atherosclerosis. Both the *PLA2G2A* and *PLA2G5* genes have been recently re-sequenced in an effort to catalogue all the common variants within both genes. This enabled through the use of haplotype algorithms such as PHASE and STRAM, the identification of a suitable set of tSNPs that potentially explained a large part of the genetic variation in both genes.

Taking into account those results for the *PLA2G2A* gene, individual tagging SNPs showed strong associations with sPLA2 IIA mass levels. When the six common haplotypes were considered there were significant differences in sPLA2 IIA mass levels between the most common haplotype (H1) and four other haplotypes ($p<0.007$). However, there was no significant association of any haplotype with intermediate phenotypes relating to lipid or inflammatory markers. This was unfortunate since evidence has previously shown that sPLA2 IIA can have multiple effects on LDL particle size, density and susceptibility to lipid peroxidation (Leitinger et al., 1999; Hurt-Camejo and Camejo, 1997; Sartipy et al., 1999; Hakala et al., 1999; Gorshkova et al., 1996; Kleinman et al., 1988; Menschikowski et al., 1995). Despite this, the strong association of sPLA2 IIA mass measures with the inferred haplotypes is a first step in establishing the causality of the sPLA2 IIA enzyme in disease. Further work is needed in order to ascertain the precise association of these haplotypes with CHD risk, since the UDAC study was not powered to investigate this association.

In addition to the analysis of the *PLA2G2A* haplotypes, it was also possible to use the Environmental Genome Project website to determine tSNPs for the closely related *PLA2G5* gene. Limited functional or epidemiological analysis has been conducted on this gene, although recent research has suggested that sPLA2 V is an effective enzyme at hydrolysing phospholipid membranes (Pruzanski et al., 2005), and can promote foam cell formation through the aggregation and fusion of lipoprotein particles (Boyanovsky et al., 2005; Murakami and Kudo, 2003; Wooton-Kee et al., 2004). When considering the individual selected tSNPs, there were significant associations with LDL, cholesterol and TG levels. Subsequent haplotype analysis revealed 7 common haplotypes accounting for 92% of the genetic variation in the UDAC study. There were significant differences in cholesterol, LDL, TG and oxLDL/LDL levels between the most common haplotype, and several other rarer haplotypes. These highly significant associations are hard to interpret

because of the lack of sPLA2 V mass measures, since these would help determine whether the effects associated with these haplotypes are a consequence of an alteration in plasma sPLA2 V mass. However, a potential mechanism could be that sPLA2 V modification of lipoproteins leads to the retention of LDL particles within the intima and the uptake of entire particles by the surface transfer process. This would then result in a faster clearance of cholesterol from the blood stream (Jaross et al., 2002), explaining some of the alterations in cholesterol, TG, and LDL seen in UDACS. Further work is needed to develop a commercially available assay as well as genotyping these tSNPs in a study that would enable an association with CHD risk to be thoroughly tested.

This section of my thesis has confirmed a significant association of sPLA2 IIA mass with various markers of atherosclerosis as well as CHD risk. In addition, the haplotype analysis conducted in the *PLA2G2A* and *PLA2G5* genes represents the first time that genetic variation in these two genes has been investigated with regards to atherosclerosis; and the strong impact that haplotypes in both genes had on markers of atherosclerosis suggests that both genes should be tested further to establish their causal relationship with CHD.

8.1.5 A comparison of candidate SNP and indirect association studies

This thesis has utilised two different techniques in order to investigate the causal relationship with CHD of three PLA2 family members. The relative merits of both methods have been discussed in chapters 4 and 7; however, as a concluding remark it is important to compare the successes and failures of the two techniques in light of the results of this thesis. The analysis of the *PLA2G7* gene utilised a candidate SNP approach, in particular investigating the association of the A379V variant with Lp-PLA2 activity and CHD risk. While this thesis has shown a relatively consistent association of Lp-PLA2 activity with CHD risk and other markers of disease, no such associations were observed when investigating the A379V variant. The success of any candidate SNP approach is dependent on previous functional evidence linking the variant to a functional effect, and this thesis has been successful in showing a consistent epidemiological relationship of the A379V variant with activity. However, the data contradicts previous *in vitro* work (Kruse et al., 2000). The inability to replicate previous associations of A379V genotype with CHD risk (Abuzeid et al., 2003; Ninio et al., 2004), and the contradictory data regarding Lp-PLA2 activity makes it difficult to confidently determine the causal relationship of this enzyme with disease when considering the data presented here. An alternative solution to this problem could be to try and find another variant with a more consistent effect on enzyme function or expression. The analysis used for the G-1230A SNP in this thesis has shown this to be a difficult and time consuming process with no guarantees of success. Alternatively, the lack of association between A379V genotype and CHD risk could imply that the Lp-PLA2 enzyme is not causally related to atherosclerosis; but until an accurate epidemiological assessment (using a comprehensive meta-analysis and mendelian randomisation) and functional analysis of genetic contributors to Lp-PLA2 activity (including further *in vitro* functional analysis of the A379V variant) are considered, this cannot be inferred from the results presented in this thesis.

By contrast, the analysis of the *PLA2G2A* and *PLA2G5* genes using an indirect approach provided a 'first-screen' method by which to test the causal relationship of these two enzymes with CHD. Since there was little evidence of any putatively functional variants in either gene, a candidate SNP approach would be unsuitable. By using tSNPs to mark most of the genetic variation in the gene, it is possible to greatly increase the power of the study under investigation, since several SNPs with moderate to small effects are likely to

be included together within haplotypes. By contrast, the *PLA2G7* A379V variant analysis only investigated the functional effect of one SNP (and potentially other SNPs in LD with the A379V variant). Testament to this was the statistically strong associations with markers of disease and sPLA2 mass that were found with both the *PLA2G5* and *PLA2G2A* haplotypes.

However, there are also significant challenges presented by using tSNP and haplotype analysis, namely the identification of a functional variant. Identification of a functional variant adds information as to the relationship of the gene product with disease and can also aid in the identification of a disease mechanism. While the sPLA2 analysis presented a first opportunity to investigate the epidemiological relationship of these enzymes with CHD, a more accurate assessment of causality could be achieved if a truly functional variant for either sPLA2 enzyme was found. Finding the functional variant using a tSNP approach can be difficult unless a single SNP differentiates two haplotypes, in which case one can investigate all those SNPs that are in strong LD with that particular tSNP. In the case of both sPLA2 enzymes, no single tSNP was responsible for the differences observed between haplotypes, therefore complicating the search for a functional variant. An additional problem found with this type of indirect analysis is the time/cost of genotyping studies for multiple variants. The use of the EGP site meant that the time and cost incurred by analysing the sPLA2 genes in this way was considerably reduced because it was not necessary to re-sequence the gene. Further characterisation of the genome in this way, along with a more detailed HapMap should enable more research of this type. The development of high throughput genotyping using techniques such as Taqman has also aided the use of haplotype analysis, but a cost differential still exists between only genotyping a select number of putatively functional variants and genotyping a relatively large number of tagging SNPs.

Both techniques have proved to be relatively successful in this thesis when investigating the contribution of these three enzymes to atherosclerosis. In some regards the different techniques are a reflection of the level to which all three genes have been studied previously. The *PLA2G7* gene has been well characterised with regards to putatively functional variants, therefore by-passing the need for a haplotype based approach. By contrast, the *PLA2G2A* and *PLA2G5* genes are at an early stage of genetic investigation, warranting the use of a tagging SNP based technology. As discussed, both techniques are

susceptible to weaknesses but currently provide one of the most cost effective ways of investigating the relationship of genes with the progression of a complex disease such as atherosclerosis.

8.1.6 The benefit of hindsight

Lp-PLA2

One of the largest problems encountered in this thesis has been with those studies investigating the relationship of Lp-PLA2 activity with intermediate phenotypes and CHD risk. The Lp-PLA2 activity assay only measures the ability of Lp-PLA2 to hydrolyse PAF, which may not relate to other phospholipid substrates (in particular oxidised PC) that could be more physiologically relevant. In addition, any significant associations could have been the result of considerable confounding from LDL. It may have been more suitable to measure Lp-PLA2 mass as well in these studies, and generate a specific activity measure, as demonstrated in the subset analysis from the NPHS II study. Specific activity would account for some of the confounding effect of LDL, since the measure relate to activity per unit of mass. Therefore, alterations in LDL levels and subsequent changes in the mass of Lp-PLA2 enzyme in circulation could be accounted for, removing a large part of the confounding effect. In addition, a comparison of the suitability of the mass measure and/or activity measure could be made in a large sample. However, the limited supply of stored plasma in those studies investigated and extra cost prevented this type of analysis.

A second considerable problem became apparent during the analysis of the A379V functional variant. Kruse et al. had previously shown that in vitro the 379V form of the enzyme exhibited a lower substrate affinity for PAF. It became apparent in the EPIC-Norfolk and NPHS II studies that individuals homozygous for the 379V alleie were actually associated with a higher Lp-PLA2 activity. In addition, the EPIC-Norfolk study contradicted the previously reported association of the 379V allele with lower CHD risk. The reasons for this remain unclear, but could relate to other important functional variants in the PLA2G7 gene not being genotyped. With the benefit of hindsight, it may have been better to conduct a full SSCP and sequencing analysis of the exons and promoter regions, as well as further research of internet SNP databases. With a full knowledge of the sequence variation in the PLA2G7 gene, one could have generated a set of tSNPs and investigated the haplotype variation with relation to Lp-PLA2 activity, intermediate phenotypes, and CHD risk. Efforts to ascertain the functional nature of the G-1230A variant could also have been combined with the analysis of other common SNPs found in the promoter of the PLA2G7 gene, and various haplotype combinations tested for their effect on PLA2G7

promoter function. These approaches may have provided a more powerful method of investigating the causal relationship of Lp-PLA2 with CHD.

A final issue that should have been addressed in this thesis relates to the expression studies of Lp-PLA2 in response to statin treatment. Firstly, the 10 and 25μM Simvastatin concentrations were selected on limited previous data. It may have been interesting to treat the cells at lower concentrations to determine the minimum Simvastatin concentration at which PLA2G7 expression is inhibited. The author did consider this possibility, but the limited yield of cultured macrophages prevented a suitable analysis. Secondly, to confirm the observation of reduced PLA2G7 gene expression after 48 hours treatment, it may have been beneficial to have assayed the cell culture medium for Lp-PLA2 activity and/or mass.

PLA2G2A and PLA2G5

The tSNPs developed for both the PLA2G2A and PLA2G5 genes were obtained from the NIEHS EGP website. The SNP information given in this website comes from a mixed population of different ethnic groups. For reasons already explained, this may have led to an incorrect assumption about the pattern of LD in these genes. Recently, the EGP website has provided separated information based on ethnic origin, and it may have been wise to revise the selected tSNPs on the basis of this information. Using other resources such as HapMap may have also shown whether the tSNPs selected were accurate across a range of different population samples. Despite this oversight, the tSNPs selected from the PDR90 population generated similar haplotype frequencies in the UDAC study, suggesting that these tSNPs were suitable for further analysis. A second potential analysis that wasn't carried out was the investigation of haplotype patterns across different ethnic groups in the UDAC study. This would have given clues as to which SNPs were consistent across common haplotypes in different populations and helped determine if any tSNP was functional or in LD with a functional variant.

Finally, it may have been useful to have genotyped the tSNPs in a large case-control study. This would have given an indication of the causal relationship of the sPLA2 enzymes with CHD risk, as well as providing data regarding haplotype frequencies in different population samples. This type of analysis is now being considered through a new grant application.

8.1.7 In conclusion

This work provides novel insight and new fundamental understanding of the role of three PLA2 enzymes in atherosclerosis at a population genetics and cellular level. This thesis has tried to determine if there is a causal relationship between Lp-PLA2, sPLA2 IIA and sPLA2 V enzymes with CHD. In the case of Lp-PLA2 a casual relationship has not been firmly established using population genetics, but has provided a large amount of data that should enable an accurate assessment of causality to be made through meta-analysis and mendelian randomisation approaches. In addition, a novel relationship of *PLA2G7* gene expression and statin therapy has been observed at the cellular level, suggesting that statins are an effective drug for the lowering of Lp-PLA2 levels directly. Attempts to further characterise genetic variation in the promoter of the *PLA2G7* gene led to the discovery of a novel G-1230A SNP, although this variant does not appear to have a functional effect regarding *PLA2G7* gene expression. The use of an indirect tagging SNP approach was successfully applied for the first time to the *PLA2G2A* and *PLA2G5* genes. This resulted in significant associations of common haplotypes in both genes and markers of atherosclerosis. Hopefully this thesis has further advanced the characterisation of three important phospholipase A2 enzymes, and will help determine if these enzymes represent suitable therapeutic targets for the treatment of coronary heart disease in the future.

8.2 Future work

8.2.1 Lipoprotein-associated PLA2

Further characterisation of the A379V variant

This thesis has highlighted the association of the 379V allele with higher plasma Lp-PLA2 activity. However, previous *in vitro* work suggested that the 379V form of Lp-PLA2 exhibited a lower substrate affinity for PAF (Kruse et al., 2000). This discrepancy needs to be addressed at a molecular level in order to determine the precise relationship of this variant with enzyme function. Recombinant protein of each genotype could be analysed with respect to their ability to hydrolyse different substrates that occur on the lipoprotein phospholipid bi-layer such as; PC, oxPC and PAF. The effect of HDL and LDL associated Lp-PLA2 activity could also be investigated, enabling a more accurate assessment of A379V functionality. This would in turn inform those associations made in this thesis and other published data concerning this variant. A longer term aim could be to use X-ray crystallography to determine if the A379V variant induces any significant changes to the enzymes active site, although the lipophilic nature of the Lp-PLA2 enzyme may prevent crystallisation of the Lp-PLA2 protein.

Testing the functionality of the promoter variants

Now that 1.9kb of promoter has been successfully cloned and transfected into cells known to express Lp-PLA2, an achievable next objective would be to investigate all the current promoter variants (T-403C, C-209G) in the *PLA2G7* gene in order to determine if any of these were functional. In particular, investigating potential haplotype combinations of SNPs may provide information as to the best SNPs to genotype in a study, and also provide an alternative functional SNP to that of the A379V variant. Sections of the 3' UTR of the *PLA2G7* gene could also be cloned into the pGL3-Basic vector enabling analysis of any variants within this region.

Development of specific activity measures

This thesis has shown that specific activity may be more informative when looking at plasma measures of Lp-PLA2 activity in epidemiological studies. Much of the previously published data contains either Lp-PLA2 activity or mass measures. Since both assays are simple to undertake and relatively low cost this may be an achievable aim in those studies

with stored plasma or serum. Our laboratory, in collaboration with our collaborators, is considering a long-term proposal to measure Lp-PLA2 mass in the NPHS II, EPIC-Norfolk and UDAC studies, with the aim of using these measures to further investigate the relationship of *PLA2G7* genotype, Lp-PLA2 specific activity and CHD risk.

Meta-analysis and the use of Mendelian randomisation

The analysis of several large prospective and case-control studies provided by this thesis should enable a large scale meta-analysis of studies that have related *PLA2G7* genotype, Lp-PLA2 activity and CHD risk in the same cohort. Previously published data from other studies such as AtheroGene may also be included upon the approval of principle investigators. The meta-analysis of publicly available data should then lead to an accurate assessment of Lp-PLA2 causality through the use of Mendelian randomisation.

Return to Haplotype analysis?

Of course there is also the possibility of taking a different approach to the direct candidate SNP analysis that has been used in this thesis. With the advent of HapMap it has become feasible to quickly determine tSNPs for use in haplotype analysis. By using this approach for the *PLA2G7* gene, a more consistent and better powered association of *PLA2G7* genetic variation with CHD risk and Lp-PLA2 activity may be achieved. However, this approach could fail to identify the causal variant, and might provide little additional information regarding the causality of this enzyme in relation to CHD.

8.2.2 Secretory PLA2

Confirming associations seen in UDACS and assessing contribution to CHD risk

The associations seen in the UDAC study represent an initial investigation into the concept of using haplotype analysis to investigate associations of the sPLA2 genes with intermediate phenotypes that could be mediated by the actions of sPLA2 *in vivo*. There is a clear need to further this investigation by genotyping similar studies to confirm the associations seen in this thesis. Linkage disequilibrium can vary across different populations (Suh and Vijg, 2005), therefore there needs to be an assessment of whether the tSNPs chosen in the UDAC study are representative of other population groups. Additionally, the UDAC study was not able to accurately assess CHD risk. Genotyping of large, well powered, and homogeneous populations for the selected tSNPs (such as EPIC-Norfolk) will allow an accurate assessment of the relationship of the common haplotypes in both genes with CHD risk. In addition, larger studies may also enable rarer haplotypes to be considered, extending the investigation of genetic variability in the *PLA2G2A* and *PLA2G5* genes.

Development of an accurate sPLA2 IIA activity assay and sPLA2 V mass assay

The development of an accurate and low cost sPLA2 IIA activity assay could potentially add further information to any epidemiological studies investigating this enzyme. However, a more pressing objective is an accurate assay for the sPLA2 GV protein. Despite the development of an antibody specific to sPLA2 GV by Cayman (www.caymaneurope.com), an effective ELISA mass assay may prove difficult to develop. As already discussed in this thesis, the sPLA2 GV enzyme is an acute phase reactant, and under normal physiological conditions plasma levels of the protein may be below the detection limit of most ELISA systems.

Use of mendelian randomisation

In a similar way to the analysis conducted for the *PLA2G7* gene, a large set of well powered studies need to be genotyped for the relevant tSNPs in the *PLA2G2A* and *PLA2G5* genes, and relevant activity/mass measures taken. Apart from confirming any preliminary observations, this would enable a meta-analysis of the available data and a thorough test of causality using Mendelian randomisation.

Searching for a functional variant

The haplotype analysis used in this thesis was unable to identify a single tSNP responsible for the significant differences in intermediate phenotypes seen between common haplotypes. The identification of a functional SNP would significantly aid the analysis of both sPLA2 enzymes in their relationship with atherosclerosis. An initial effort could be made to see if any of the common variants identified in both genes were responsible for changes in the amino acid sequence of the protein. If any of the variants did result in a non-synonymous change, then genotyping in an epidemiological study could confirm if the change resulted in significant differences in enzyme activity or mass. Creation of a recombinant protein would also enable an *in vitro* investigation of the altered protein's properties. Any variants within the promoter or 3' UTR regions may disrupt protein-DNA binding sites and could be investigated using the dual luciferase reporter system described in this thesis. A more complex epidemiological method to determine the functional SNP could be to use the differences in LD that exist between different population groups. The difference in LD and haplotype structure between different ethnic groups may give a clue to which SNP or SNPs within a haplotype of interest could be influencing the observed association. This relatively new concept, provides an alternative method to using 'best estimate' approaches to find the relevant functional SNP.

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Tagging-SNP haplotype analysis of the secretory PLA_2IIa gene *PLA2G2A* shows strong association with serum levels of sPLA2IIa: results from the UDACS study

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Recent prospective analysis identified secretory phospholipase A_2-IIa ($sPLA_2$IIa) as a coronary artery disease (CAD) risk predictor. This study aimed to examine the relationship between serum levels of $sPLA_2$IIa and variation in the $sPLA_2$IIa gene (*PLA2G2A*) in a cohort of patients with Type II diabetes (T2D) mellitus. Six tagging single nucleotide polymorphisms (tSNPs) accounting for >92% of the genetic variability in *PLA2G2A* were identified and distinguished six common haplotypes (frequencies >5%). In the 523 Caucasian T2D patients, levels of $sPLA_2$IIa, independent of CRP, were negatively correlated with total antioxidant status ($P = 0.003$) and high-density lipoprotein cholesterol ($P = 0.006$) in men and correlated with CAD status in women ($P = 0.002$) (Odds ratio of top two tertiles versus bottom = 2.50) [95% CI (1.13–5.53) $P = 0.024$]. Overall, tSNP haplotypes showed a highly significant association with $sPLA_2$IIa levels ($P < 0.0001$), explaining 6.3% of the variance. The most common haplotype (frequency 14.2%) was associated with 53% higher $sPLA_2$IIa levels [3.25 ng/ml ($\pm$0.14)] compared with the combined other haplotypes [2.13 ng/ml ($\pm$0.09), $P < 0.00001$]. Five of the six tSNPs were associated with significant effects on $sPLA_2$IIa levels but the raising haplotype could not be distinguished by a single tSNP and none are likely to be functional. These data confirm the relationship between elevated $sPLA_2$IIa levels and CAD risk reported in both cases: control and prospective analyses. The strong impact of *PLA2G2A* haplotypic variation on $sPLA_2$IIa levels will help clarify the causality of this association.

INTRODUCTION

Secretory phospholipase A2 group IIa ($sPLA_2$IIa) is a member of a superfamily of enzymes that hydrolyse the *sn-2* ester bond of phospholipids and cell membranes, generating non-esterified free fatty acids (NEFAs) and lysophospholipids (1). $sPLA_2$IIa is expressed in many cell types and may be an enzymatic component of the host defence mechanism directed against bacterial invasion and part of the inflammation-associated cellular responses (2). In addition, and more relevant to this study, the presence of $sPLA_2$IIa activity in the arterial wall suggests that it may play a role in atherogenesis (3).

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In fact sPLA$_2$IIa has been shown to have pro-atherogenic properties both in the circulation and within the arterial wall (3). In the circulation, sPLA$_2$IIa hydrolysis of low-density lipoprotein (LDL) generates oxidation susceptible, small-dense LDL (sd-LDL) particles, with altered configuration of apolipoprotein B (4), leading to LDL receptor (LDLR) independent uptake, promoting atherogenesis (5). In the process of hydrolysing LDL, both lysophospholipids (precursors of pro-inflammatory mediators of leukotrienes and prostaglandins) (6) and NEFAs, including arachadonic acid, can be released, with downstream pro-inflammatory consequences (7). Acute phase high density lipoprotein (HDL) is a better substrate for sPLA$_2$IIa than normal HDL, suggesting a possible role in the inflammatory response and in the metabolism of acute phase lipoproteins (8).

In the arterial wall, sPLA$_2$IIa-modified lipoproteins show increased susceptibility to lipid peroxidation (9), producing oxidized lipoproteins that enhance macrophage growth (10). These modified lipoproteins bind more tightly to extra-cellular proteoglycans, which leads to their enhanced retention in the arterial wall (4,11,12), an early marker of atherogenesis (13). The products of sPLA$_2$IIa hydrolysis, oxidized NEFAs and lysophosphatidylcholine, induce further aggregation and fusion of lipoproteins, leading to accumulation within the extra-cellular matrix and eventual internalization in macrophages within the intima (14). Although immunohistochemical studies have identified sPLA$_2$IIa in normal arteries, its extra-cellular distribution and cell expression is increased in early and late atherosclerotic lesions, further implicating it in atherosclerosis (15–17). The pro-atherogenic role of sPLA$_2$IIa has been confirmed in mouse models, and mice transgenic for the human sPLA$_2$IIa gene (*PLA2G2A*) show a dramatic increase in atherosclerosis, on both high fat and chow diets (18,19). These *PLA2G2A* transgenic mice in addition had raised LDL- and total cholesterol, and sPLA$_2$IIa was present in the atherosclerotic plaques on the surface of macrophages. To identify whether this was the source of increased atherosclerosis, bone marrow from *PLA2G2A* transgenic mice was transplanted into *ldlr*−/− mice fed a high fat diet. There was no effect on plasma lipoprotein levels, however, mice showed an increase in the extent of atherosclerosis, suggesting that the macrophage-expressed sPLA$_2$IIa contributed to the lesion formation (20).

sPLA$_2$IIa is an acute phase protein expressed in response to a variety of pro-inflammatory cytokines (3,21,22). Circulating levels of sPLA$_2$IIa are higher in coronary artery disease (CAD) patients compared with apparently healthy individuals (23,24) and are associated with increased risk of future CAD, in prospective analysis (25), but whether this is a causal relationship remains to be determined.

In this study, we have examined the hypothesis that serum sPLA$_2$IIa levels are significantly determined by variation in the sPLA$_2$IIa gene, *PLA2G2A*, using a tagging single nucleotide polymorphism (tSNP) approach. These tSNPs can infer the allelic state of all the common SNPs in the gene with a high coefficient of determination, thus covering maximum genetic variability. The study cohort was composed of patients with Type II diabetes (T2D), a group with increased CAD risk, with well-characterized measures of oxidative stress and LDL particle size.

Table 1. Baseline characteristics (mean and SD) of Caucasian patients with T2D from UDACS

	No CAD $n = 383$	CAD[a] $n = 136$	P-value
Age (years)	65.5 (11.3)	69.5 (9.7)	0.0003
BMI (kg/m^2)[b]	29.2 (5.5)	29.5 (4.7)	0.67
HbA1c (%) [b]	7.7 (1.7)	7.5 (1.5)	0.27
Glucose (mmol/l)[b]	10.02 (4.40)	9.58 (4.25)	0.31
Cholesterol (mmol/l)	5.19 (1.07)	4.71 (1.12)	<0.0001
LDL (mmol/l)[c]	2.81 (0.93)	2.32 (0.89)	<0.0001
HDL (mmol/l)[b]	1.30 (0.38)	1.23 (0.37)	0.06
TG (mmol/l)[b]	1.90 (1.06)	1.92 (1.07)	0.84
SBP (mmHg)[b]	141.5 (20.6)	140.0 (20.9)	0.47
DBP (mmHg)	81.2 (11.4)	78.4 (10.0)	0.01
Duration of diabetes (yrs)[d]	8 [4 - 16]	11 [6 - 17]	0.005
Gender (% male)	57.2% (219)	66.2% (90)	0.07
Smoking (% current)	17.0% (64)	12.0% (16)	0.18
TAOS (%)[d]	44.9 [36.7 - 52.5]	42.9 [34.1 - 50.7]	0.13
Ox-LDL/LDL (U/mmol)[b]	16.8 (7.8)	18.6 (10.3)	0.08
PPD/MPD (nm)[e]	0.991 (0.016)	0.993 (0.018)	0.35
Sd-LDL (%)[d]	71.9 [58.5 - 81.4]	71.5 [54.9 - 80]	0.74
CRP (mg/l)[b]	1.66 (1.42)	1.77 (1.59)	0.49
sPLA$_2$IIa (ng/ml)[b]			
Total	3.08 (2.20)	3.45 (2.62)	0.12
Women	3.81 (2.66)	4.47 (2.89)	0.17
Men	2.62 (1.81)	3.02 (2.36)	0.12
Statin (%)	23.0	60.0	<0.0001
ACE inhibitor (%)	26.5	38.9	0.003
Aspirin (%)	21.6	43.4	<0.0001

[a]$n = 7$ had missing CHD data.
[b]Log-transformed.
[c]Square root transformed.
[d]Median [IQR].
[e]Peak partial diameter LDL/mean particle diameter LDL.

RESULTS

Table 1 summarizes the baseline characteristics of the Caucasian men and women with T2D, in relation to the presence/absence of CAD. Those who had CAD were significantly older and had a longer duration of diabetes. The higher usage of statins, angiotensin converting enzyme (ACE) inhibitors and aspirin potentially explains their lower diastolic blood pressure (BP), LDL-C and total cholesterol levels compared with CAD-free men and women. Serum sPLA$_2$IIa levels were not statistically significantly different comparing those with CAD and those free of CAD. However, compared with the men, women had significantly higher sPLA$_2$IIa levels (3.94 and 5.96 ng/ml, respectively, $P < 0.0001$). In the men, sPLA$_2$IIa levels were significantly negatively correlated with total antioxidant status (TAOS) and HDL, independent of age, CAD status, statin use and CRP and were positively correlated with CRP after adjustment for age, CAD status and statin use (Table 2A). In the women, the proportion of CAD events significantly correlated with sPLA$_2$IIa, even after adjustment for age, statin use and CRP, with women in the top two tertiles having statistically significantly higher sPLA$_2$IIa levels than those in the bottom tertile, [Odds ratio (OR) of top two tertiles versus bottom tertile = 2.50; 95% CI (1.13–5.53) $P = 0.024$ unadjusted and 4.82 (1.79–13.0) $P = 0.002$ after adjustment] (Table 2B). Although TAOS was negatively correlated with sPLA$_2$IIa ($P < 0.006$), this did not remain statistically significant after adjustment.

Table 2. Distribution of the coronary heart disease and diabetes risk factors (mean and SD) according to $sPLA_2$ tertiles in Caucasian men (A) and women (B) with T2D

		Tertile of $sPLA_2$-IIa (ng/ml)			*P*-value for ANOVA		
		1	2	3	Unadjusted	Adjusted[a]	Adjusted[b]
		<1.95	1.95–3.30	>3.30			
		n = 107	*n* = 102	*n* = 104			
(A)	TAOS (%)[c]	48.6 [38.6–56]	42.2 [31.6–51.2]	41.2 [35–47.6]	0.0002	0.04	0.003
	BMI (kg/m^2)[d]	29.0 (5.0)	29.6 (5.2)	29.4 (5.1)	0.65	0.29	0.68
	LDL (mmol/l)[e]	2.56 (1.07)	2.67 (0.96)	2.56 (0.83)	0.69	0.54	0.36
	HDL (mmol/l)[d]	1.25 (0.37)	1.20 (0.33)	1.11 (0.28)	0.009	0.003	0.006
	Ox-LDL/LDL (U/mmol)[d]	18.9 (9.7)	15.1 (7.1)	18.7 (8.8)	0.01	0.01	0.01
	Sd-LDL (%)[c]	65.9 [50.1–77.6]	74.7 [62.7–81.4]	71.7 [59.0–82.5]	0.05	0.65	0.26
	PPD/MPD (nm)[f]	0.990 (0.017)	0.992 (0.015)	0.991 (0.017)	0.76	0.79	0.78
	Cholesterol (mmol/l)	5.04 (1.22)	4.90 (1.03)	4.73 (1.03)	0.12	0.27	0.25
	Smokers (%)	13.5 (14)	15.0 (15)	19.4 (20)	0.48	0.31	0.48
	CRP (mg/l)	1.32 (1.02)	1.49 (1.32)	2.04 (1.72)	0.0007	0.0004	—
	CAD (%)	25.0 (26)	29.4 (30)	33.0 (34)	0.45	0.68	0.91
	Odds ratio (95% CI)	1.00	1.25 (0.68–2.31)	1.48 (0.81–2.71)	0.21	0.39	0.66
	HbA1c (%)[d]	7.83 (1.70)	7.24 (1.47)	7.58 (1.54)	0.03	0.03	0.08
	Glucose (mmol/l)[d]	10.41 (4.40)	8.99 (4.07)	9.82 (4.32)	0.06	0.08	0.10
(B)	TAOS (%)[c]	49.4 [37.9–56.5]	44.0 [35.4–49.5]	41.2 [31.8–48.7]	0.009	0.06	0.65
	BMI (kg/m^2)[d]	29.3 (5.9)	28.7 (5.7)	29.9 (6.3)	0.44	0.33	0.72
	LDL (mmol/l)[e]	2.83 (0.90)	2.84 (1.04)	2.74 (0.77)	0.76	0.51	0.50
	HDL (mmol/l)[d]	1.47 (0.40)	1.42 (0.35)	1.43 (0.44)	0.78	0.70	0.77
	Ox-LDL/LDL (U/mmol)[d]	18.5 (6.3)	15.8 (7.8)	15.8 (9.4)	0.18	0.24	0.25
	Sd-LDL (%)[c]	68.2 [55.7–76.2]	74.1 [61.7–82.4]	75.0 [58.4–84.5]	0.03	0.25	0.07
	PPD/MPD (nm)[f]	0.993 (0.017)	0.992 (0.019)	0.992 (0.013)	0.96	0.97	0.82
	Cholesterol (mmol/l)	5.26 (1.04)	5.38 (1.24)	5.29 (0.87)	0.81	0.63	0.54
	Smokers (%)	12.7 (9)	13.2 (9)	18.3 (13)	0.58	0.46	0.42
	CRP (mg/l)[d]	1.58 (1.32)	1.66 (1.59)	2.34 (1.96)	0.02	0.02	—
	CAD (%)	12.7 (9)	31.3 (21)	22.2 (16)	0.03	0.01	0.002
	Odds ratio (95% CI)	1.00	3.14 (1.32–7.50)	1.97 (0.81–4.81)	0.17	0.06	0.02
	HbA1c (%)[d]	7.50 (1.73)	7.88 (1.63)	8.06 (1.58)	0.12	0.03	0.08
	Glucose (mmol/l)[d]	9.94 (4.49)	9.54 (4.16)	10.95 (4.59)	0.16	0.15	0.36

[a]Adjusted for age, CAD status and statin use.
[b]Adjusted for age, CAD status, CRP and statin use.
[c]Median [IQR].
[d]Log-transformed.
[e]Square root transformed.
[f]Peak particle diameter LDL/mean particle diameter LDL.

Using a web-based database with complete resequencing data for *PLA2G2A* (egp.gs.washington.edu/data/pla2g2a/pla2-g2a.genotyping.html), six tSNPs were identified which together explained >92% of the haplotype variability in *PLA2G2A* (Table 3). The location of these tSNPs [one in the promoter region, two silent SNPs in the coding sequence (exons 2 and 4), one in intron 3 and two in the 3′-untranslated region, UTR] is presented in Figure 1, together with their pair-wise linkage disequilibrium (LD). The tSNPs fall into three LD blocks.

The univariate analyses of the tSNPs with intermediate phenotypes are presented in Supplementary Material, Table S2 (A–F). Four out of the six tSNPs (−655T > C, 763C > G, 1983G > A, 5128T > G) showed strong associations with $sPLA_2$IIa levels only ($P < 0.0001$), whereas with a fifth SNP (1022G > T) the association was less strong ($P = 0.01$). In addition, −655T > C showed strong association with LDL cholesterol levels ($P = 0.007$). However, as these SNPs were primarily identified for tagging purposes, i.e. chosen to cover the genetic variability of the gene, only the haplotype analysis is considered in detail here. Of the potential

Table 3. Reference SNP (rs) number and minor allele frequency of the tSNPs in *PLA2G2A*, used in the study

tSNP	rs number	Minor allele frequency (95% CI)
−655T > C	rs1774131	0.33 (0.30–0.36)
763C > G	rs11573156	0.23 (0.20–0.25)
1022G > T	rs3753827	0.45 (0.42–0.47)
1983G > A	rs2236771	0.11 (0.09–0.13)
4982T > A	rs876018	0.17 (0.15–0.19)
5128T > G	rs3767221	0.39 (0.36–0.42)

64 haplotypes defined by six tSNPs, 23 inferred haplotypes were observed in the sample. Of these haplotypes, six occurred at frequencies >5% and accounted for 68% of the observed haplotypes. Eleven tSNP haplotypes occurred at frequencies between 1 and 5% and six occurred at frequencies <1% (see Supplementary Material, Table S3). The frequencies and associated $sPLA_2$IIa levels for the six haplotypes occurring at frequencies >5% are presented in Table 4. Overall,

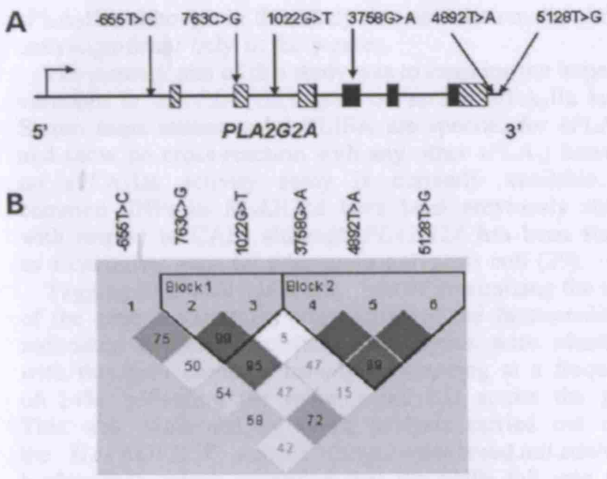

Figure 1. (**A**) Map of the $sPLA_2$ (*PLA2G2A*) gene showing the exons (filled boxes) and introns (hatched boxes) and the position of the six tSNPs, numbered from the start of exon 1. (**B**) *HAPLOVIEW* LD (*D'*) display of tSNPs of *PLA2G2A*. The darker the box the stronger the LD. The *D'* LD for any two tSNPs is presented in the box representing their intersection. No number denotes complete LD.

Table 4. *PLA2G2A* tSNP haplotypes which occur at frequencies >5% and their association with $sPLA_2$IIa levels

Haplotype	Frequency %	$sPLA_2$ levels ng/ml (SD)[a]	*P*-value compared to CCGGAT
H1: CCGGAT	14.2	3.26 (3.25)	
H2: TGGGAG	13.3	2.21 (4.35)	<0.0001
H3: TGTGAT	13.1	2.07 (20.9)	<0.002
H4: TGTGTT	11.3	2.15 (2.00)	<0.007
H5: TGTGAG	8.0	1.80 (2.08)	<0.001
H6: TGGGAT	7.7	3.08 (3.99)	0.65

Overall $P < 0.0001$.
[a]Mean value for one copy of haplotype (unadjusted).

haplotypic variation in *PLA2G2A* was associated with a highly significant effect on $sPLA_2$IIa levels ($P < 0.0001$). The most frequently occurring haplotype H1 (CCGGAT) was associated with 53% higher $sPLA_2$IIa levels [3.26 ($\pm$0.14) ng/ml] than all the other five haplotypes combined ($P < 0.00001$). The $sPLA_2$IIa levels for these five haplotypes did not differ significantly from each other ($P = 0.34$) with a mean level of 2.13 ($\pm$0.13) ng/ml. Haplotypic variation in *PLA2G2A* explained 6.3% of the variance in $sPLA_2$IIa levels. These haplotypes showed no significant association with any other intermediate trait (data not shown), and there was no frequency difference of any of the haplotypes comparing those with or without CAD.

In an attempt to identify a potentially functional SNP associated with the $sPLA_2$IIa-raising effect of H1, a cladogram representing the evolutionary relatedness of the haplotypes was drawn up using the website http://www.fluxus-engineering.com (Fig. 2). H1 is separated from H6 by two changes, whereas H2 and H3 are separated from H6 by a single change

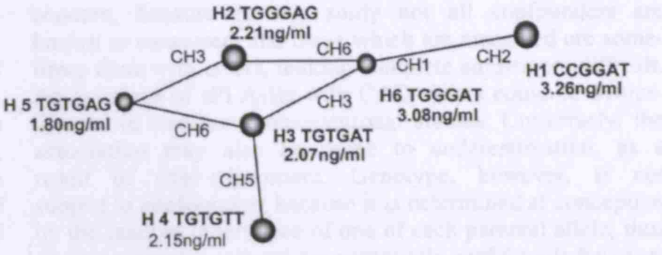

Figure 2. Tagging-SNP cladogram for *PLA2G2A*. The diameters of the circles representing observed haplotypes are drawn relative to their haplotype frequency, compared with H1. Text along the line represents the changes that have occurred which separates one haplotype from the other, e.g. CH6 represents a change in tSNP 6. When more than one change has occurred the order on the figure is arbitrary.

each. H5 could be derived from either H2 or H3 by a single change, whereas H4 is separated from H3 by a single change. Thus no single SNP could distinguish H1 from the other haplotypes to suggest a single-functional SNP.

DISCUSSION

In this study, we report the strong association of *PLA2G2A* tSNP haplotypes with serum $sPLA_2$IIa levels. The University College London Diabetes and Cardiovascular (UDACS) study was chosen for this genetic analysis of *PLA2G2A* because of the detailed measures of oxidative stress and LDL size, variables that are known to be influenced by $sPLA_2$, and which might show association with *PLA2G2A* variation. As reported previously in the EPIC study (25), in this present study, women had significantly higher $sPLA_2$IIa and CRP than men. These higher levels in EPIC were independent of hormone replacement therapy (25). In women, $sPLA_2$IIa levels correlated with CAD risk, with an OR of 2.50 [95% CI (1.13–5.53) $P = 0.02$] in the top two tertiles combined, compared with the bottom tertile, which remained significant even after adjustment for CRP ($P < 0.0002$). In the men this trend was evident, but did not reach statistical significance. In the men, $sPLA_2$IIa levels showed strong negative correlation with HDL cholesterol, seen previously in the EPIC study, and with TAOS, a net measure of plasma-oxidative stress, with higher TAOS, equating to lower the oxidative stress. Although TAOS is not a highly specific measure of plasma-oxidative stress, for a large number of samples it is a practical, inexpensive assay and there is a evidence supporting the use of plasma TAOS as a marker of plasma-oxidative stress (26) with, for example, a strong correlation ($r = -0.65$) between the plasma TAOS and the gold-standard measure of oxidative stress, esterified F2-isoprostane levels ($P = 0.003$) (27). The inverse relationship between $sPLA_2$IIa and TAOS is supported by a recent study demonstrating that macrophage-specific over-expression of $sPLA_2$IIa, after bone marrow transplantation from $sPLA_2$IIa transgenic mice, accelerated atherogenesis in *ldlr*−/− mice, with an increase in oxidative stress as measured by F2-isoprostanes (28). Thus our results add weight to the concept that the increased-oxidative stress associated with high $sPLA_2$IIa levels provides an additional mechanism for the pro-atherogenic role of

sPLA$_2$IIa, although in this study this association was statistically significant only in the women.

The primary aim of this study was to examine the impact of variation in the *PLA2G2A* gene on serum sPLA$_2$IIa levels. Serum mass measures by ELISA are specific for sPLA$_2$IIa and show no cross-reaction with any other sPLA$_2$; however, no sPLA$_2$IIa activity assay is currently available. No common SNPs in *PLA2G2A* have been previously studied with respect to CAD, although *PLA2G2A* has been studied as a candidate gene for ademtous polyposis coli (29).

Tagging-SNP analysis is a method of maximizing the study of the genetic variability irrespective of the functionality of individual SNPs. Six common haplotypes were identified, with the most common haplotype occurring at a frequency of 14%, reflecting the rather weak LD across the gene. This was confirmed by block analysis carried out using the *HAPLOVIEW* website (http://www.broad.mit.edu/mpg/haploview/), which identified that the SNPs fall into three LD blocks. Overall, the haplotypes were associated with highly significant effects on sPLA$_2$IIa levels, $P < 0.00001$, confirming that the variation in *PLA2G2A* was contributing to the sPLA$_2$ variance. This contribution to the variance did not change appreciatively (6.4%), if we included all the observed haplotypes (data not shown). Although genetic contribution of *PLA2G2A* to the variance in sPLA$_2$IIa levels is relatively low, it is in the same order of magnitude as the variance in cholesterol ester transfer protein (CETP) concentration explained by functional variants of *CETP* (30). Frisdal *et al.* (30) reported that the two-functional SNPs in the CETP promoter which explain the intronic TaqIB association, namely $-629C > A$ and $-1137C > T$, explain 10.1% of the variance in CETP concentration.

The common *PLA2G2A* haplotype, H1, was associated with 53% higher sPLA$_2$IIa levels compared with the pooled other haplotypes, which suggests that this haplotype might be associated with increased CAD risk. However, its low frequency and the relatively small sample size in UDACS mean that this study is underpowered to address the question.

The cladogram analysis carried out provides an unrooted evolutionary tree (31) to attempt the identification of a functional SNP(s), or in the case of tSNPs, the 'bin' in which the functional SNP might occur. Haplotype H1 differs from H2 (and all the four other haplotypes) by the presence of the minor and common alleles, respectively, of 5′-variants $-655C/763C$ compared with $-655T/763G$ present in the other five haplotypes. No other single SNP was unique to H1 that could explain the sPLA$_2$IIa-raising effect. This suggests that there is more than one-functional SNP accounting for this sPLA$_2$-raising effect and further genotyping will be required to identify this.

Several studies (23–25) have found an association between sPLA$_2$IIa levels and CAD risk, however, as with the association of lipoprotein associated-PLA$_2$ and risk (32–34), causality has not been formally established (35). In traditional epidemiological studies, the association between phenotype (a risk factor) and disease is often biased by confounding and reverse causation, i.e. the raised-risk factor is a result of the disease state not causal of it (36,37). Although statistical adjustment makes some allowance for confounding, residual confounding is a concern, because in any study not all confounders are known or measured, and those which are measured are sometimes done with errors, making complete adjustment difficult. Associations of sPLA$_2$IIa with CAD events could be overestimated in traditional observational studies. Conversely, the association may also be prone to underestimation, as a result of over-adjustment. Genotype, however, is not subject to confounding because it is determined at conception by the random inheritance of one of each parental allele, thus common genetic variants are potentially useful tools for overcoming the confounding. If sPLA$_2$IIa really does increase the risk of CAD events, individuals with alleles that raise sPLA$_2$IIa should have an increased risk of events similar to their effect on sPLA$_2$IIa level. Moreover, if sPLA$_2$IIa really exerts effects on CAD risk through, for example, BP, then these individuals should have a higher BP. Mendelian randomization is an approach which examines the triangular relationship among genotype, phenotype and disease and enables the determination of causality (37,38). These present results strongly suggest that variation in *PLA2G2A* is having its effects through sPLA$_2$IIa levels directly, but UDACS is inadequately powered to detect an association between genotype and CAD risk. However, these studies provide the groundwork, by identifying the strong relationship between tSNP haplotypes and sPLA$_2$IIa variance, for future *PLA2G2A* haplotype investigations in large prospective or case: control studies, to determine the relationship among *PLA2G2A* genotype, serum sPLA$_2$IIa levels and CAD risk, to clarify this issue of causality.

MATERIALS AND METHODS

Study design

UDACS study consist of 1014 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) 2001–2 (629 men; 532 Caucasians with T2D). All patients had diabetes according to WHO criteria (39). Analysis was restricted to the Caucasian subjects with T2D to remove possible heterogeneity within the sample. Six of these patients had sPLA2IIa levels more than 3 standard deviations (SD) from the mean and were excluded from further analysis, as it might imply underlying infection. Thus 526 Caucasian patients with T2D (313 men and 213 women) were examined further. CAD status was not available for seven of these patients. Information about medication was available, particularly statin usage, ACE inhibitors and aspirin. Aspirin as an inhibitor of COX-1 and COX-2 affects platelet function by inhibiting the enzyme prostaglandin. sPLA$_2$IIa also feeds into the prostaglandin pathway and therefore may be ultimately affected by aspirin usage.

Clinical measurements

CAD event was recorded if any patient had positive coronary angiography/angioplasty, coronary artery bypass, cardiac thallium scan, exercise tolerance test, myocardial infarction or symptomatic/treated angina, detailed elsewhere (26). Routine plasma traits were measured (26) including plasma oxidized LDL by ELISA (Mercodia, Uppsala, Sweden), expressed as

the ratio of oxidized LDL divided by total LDL to generate a specific measure of LDL oxidation (40). Plasma TAOS, which is inversely related to oxidative stress, was measured by a photometric microassay (41). LDL particle size and peak particle diameter were measured as previously described (42,43). The percentage small-dense LDL (sd-LDL) is derived from the percentage of LDL subclasses I and II from the four subclasses I–IV obtained by ultracentrifugation (44). Serum sPLA2IIa levels were measured by a commercially available ELISA (Cayman Chemical Company, Ann Arbor, MI, USA). The intra- and inter- assay coefficients of variation were 6.0 and 10.3%, respectively. Full ethical approval was granted by the UCHL NHS Trust and all patients included in the study had consented.

DNA extraction, tagging-SNP identification and genotyping

DNA was extracted using the salting out method (45). tSNPs were identified using the STRAM algorithm (46) on the PHASE (47) output from the National Institute of Environmental Health Sciences SNP database website http://egp.gs.washington.edu/genes.html. Six tSNPs of *PLA2G2A* were identified (rs1774131, rs11573156, rs3753827, rs2236771, rs876018, rs3767221). All SNPs were genotyped using TaqMan technology (Applied Biosciences, ABI, Warrington UK). Reactions were performed on 384-well microplates and analysed using ABI TaqMan 7900HT software. Primers and MGB probes are detailed in Supplementary Material, Table S1.

Statistical methods

The Hardy–Weinberg equilibrium and the LD (D') of tSNPs were assessed using THESIAS (48,49). All analyses were performed on normally distributed data after appropriate transformation (log or square root). Results are presented as mean and SD. Parametric or non-parametric (Kruskal–Wallis) analysis of variance was used, when appropriate, to compare the changes of the continuous variables across the SNPs categories. Multiple regressions were used to calculate the adjusted R-square for the proportion of the variance explained by the model. For the categorical variables, Pearson's chi-square or Fisher's exact tests were used depending on the expected values of each category in the two-way table. Adjusted P-values were obtained from the analysis of covariance for continuous data and logistic regression for categorical data. Haplotypes were inferred using both THESIAS (48,49) and PHASE (47) excluding individuals with missing values. The haplotypic pair for each subject was calculated by PHASE (47) and only the haplotypes with frequencies >5% were used for further analysis. Because of multiple testing, the significance level was taken as $P < 0.01$, instead of an inappropriately conservative Bonferroni-like adjustment of the P-values (50,51).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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Lp-PLA2 activity and *PLA2G7* A379V genotype in patients with diabetes mellitus

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Abstract

Lipoprotein associated phospholipase A2 (Lp-PLA2) modulates low-density lipoprotein (LDL) oxidation by hydrolysing oxidised phospholipids present on particle surfaces. We investigated whether Lp-PLA2 activity and *PLA2G7* A379V genotype were related to mediators of atherosclerosis in a diabetic study. Plasma Lp-PLA2 activity (taken in men only) and A379V genotype were investigated with regards to metabolic syndrome (MS), UKPDS risk score, and oxidised LDL (oxLDL/LDL), in a cohort of Caucasian men and women ($n = 783$, age 62.5 ± 13.7 years). After adjustment for type of diabetes, CHD status, and statin use, those individuals with features defining the MS (WHO guidelines) had higher Lp-PLA2 activity (35.6 ± 11.9 nmol/min/ml) compared to those without (33.0 ± 10.8 nmol/min/ml) ($p = 0.02$). Quartiles of UKPDS coronary heart disease (CHD) risk score were also positively associated with Lp-PLA2 activity ($p = 0.006$, $p = 0.004$ linear trend). Those men in the highest quartile of oxLDL/LDL level had the lowest Lp-PLA2 activity (31.3 ± 10.5 nmol/min/ml) when compared to the middle two (32.3 ± 9.8 and 35.9 ± 10.9 nmol/min/ml, respectively) and lowest quartile (35.6 ± 12.5 nmol/min/ml; $p = 0.03$, $p = 0.004$ linear trend). There was no significant association between A379V genotype and Lp-PLA2 enzyme activity ($p = 0.34$) or oxLDL/LDL ($p = 0.32$). Lp-PLA2 activity is an independent predictor of CHD risk and MS in a sample of subjects with diabetes mellitus. The association of Lp-PLA2 activity with oxLDL/LDL suggests that Lp-PLA2 may be a modulating factor in the process of atherosclerosis.

Keywords: Lp-PLA2; PAFAH; Oxidised LDL; Metabolic syndrome; Diabetes

1. Introduction

Lipoprotein associated phospholipase A2 (Lp-PLA2) or platelet activating factor acetylhydrolase (PAFAH), is a member of the superfamily of enzymes defined by their ability to hydrolyse the middle (*sn*-2) ester bond of phospholipids [1]. Lp-PLA2 is secreted into the plasma by platelets [2], macrophages [3], and tissue sources that are consistent with the macrophage origin of the enzyme [4]. Approximately 80% of the plasma enzymatic activity of Lp-PLA2 is associated with low-density lipoprotein (LDL) particles, with the remainder residing on high-density lipoprotein (HDL) [5].

Lp-PLA2 has a marked substrate preference for choline-containing phospholipids including platelet-activating factor (PAF), and short fatty acid chain oxidised phospholipids, both of which interact with a cell surface G-protein-coupled receptor, leading to inflammatory cell activation [6]. In this context, Lp-PLA2 may play an *anti-atherogenic* role, by converting a potent inflammatory signalling molecule present in plasma into two biologically inactive metabolites, lyso-PAF and acetate [7]. However, recent evidence suggests that Lp-PLA2 may also have *pro-inflammatory* activity,

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associated with the generation of significant amounts of lysophosphatidylcholine (lyso-PC) and free oxidised fatty acids from the degradation of oxidised phosphatidylcholine (oxPC) [8].

The association of Lp-PLA2 with LDL, via an interaction with apolipoprotein (apo) B [9], makes this enzyme ideally placed to participate in the key oxidative steps of atherogenesis. Lp-PLA2 degradation of oxidised phospholipids present on the surface of LDL molecules generates significant concentrations of lyso-PC and oxidised free fatty acids [10]. However, the Lp-PLA2 mediated breakdown of oxidatively damaged phospholipids may protect LDL particles from involvement in the progression of atherosclerosis.

The opposing pro- and anti-atherogenic properties of Lp-PLA2 have been demonstrated both in human and animal models. Lp-PLA2 has been found to be expressed by macrophages in human and rabbit lesions, possibly contributing to atherosclerosis by the release of lyso-PC and free fatty acids [11]. In vitro experiments have also demonstrated that specific inhibition of Lp-PLA2 reduced by a third the cytotoxic effects of oxidised LDL on monocyte-macrophages [12]. However, adenoviral over-expression of Lp-PLA2 in *apoe* deficient mice led to reduced neointima formation, endothelial damage and spontaneous atherosclerosis [13], along with a protection of plasma lipoproteins from oxidation [14].

Epidemiological data has not been able to clarify the pro- and anti-atherogenic role of Lp-PLA2. Both activity and mass of the enzyme in Caucasian populations has been shown to be a consistent risk marker for CHD, independent of traditional risk factors [15–18], although it is still unclear whether plasma Lp-PLA2 exhibits a causal role, or is a marker of risk. A loss-of-function mutation present in 4% of the Japanese population (V279F) has been reported to be associated with an increased risk of coronary artery disease [19], supporting the *anti-atherogenic* action of this enzyme. In order to relate the variability within the Lp-PLA2 gene (*PLA2G7*) to atherosclerosis within Caucasian subjects, several variants have been detected, with the Alanine to Valine change at position 379 (exon 11) being found to be functional [20]. Subsequent in vitro studies showed a two- to three-fold decrease in the affinity of Valine 379 Lp-PLA2 recombinant protein for its substrate PAF [20]. In addition, V379 homozygotes were found to be protected from CHD in the HIFMECH case-control study [21]. The previous association of this genotype with lower Lp-PLA2 PAF substrate affinity would therefore support a *pro-atherogenic*, causative role for Lp-PLA2 in CHD.

Ninio et al. investigated the Lp-PLA2 A379V polymorphism, as well as several other known non-synonymous and promoter variants, in the Athero*Gene* study of 1318 CAD patients and 485 controls [22]. With respect to case-control status and clinical outcome, the 379V allele was found to be independently protective against the development of CAD, supporting the HIFMECH study [21]. However, in contrast to data produced by Kruse et al. [20], those homozygous for the 379V allele showed significantly *higher* plasma Lp-PLA2 activity towards hydrolysing PAF substrate compared to AA and AV individuals, suggesting an *anti-atherogenic* role for Lp-PLA2 in atherosclerosis.

We have examined a cohort of high-risk patients with diabetes mellitus in order to characterise further the relationship of Lp-PLA2 with LDL oxidation and atherosclerosis. In particular, our aim was to determine in these individuals whether Lp-PLA2 activity and A379V genotype have an effect on the plasma levels of oxidised LDL, risk of CHD, and features of the metabolic syndrome.

2. Materials and methods

2.1. Study design

The University College London Diabetes And Cardiovascular Study (UDACS) consisted of 1014 consecutive subjects recruited from the diabetes clinic at University College London Hospitals NHS Trust (UCLH) 2001-2 [629 men; 183 with type 1 diabetes mellitus; mean age 61.8 ± 13.3 years; median duration of diabetes 11 (5–19) years] [23]. All patients had diabetes according to WHO criteria [24]. Analysis was restricted to the Caucasian subjects to remove possible heterogeneity within the sample ($n = 783$). Lp-PLA2 activity was measured in male Caucasian subjects only ($n = 433$) as previous data has suggested that estrogen can affect Lp-PLA2 enzyme levels [25]. The smaller number of Caucasian women with genotype data suggested there would not be sufficient power to detect a genotype effect on activity in the women.

2.2. Definitions and clinical measurements

Individuals classified as having 'CHD' were recorded if any patient had positive coronary angiography/angioplasty, coronary artery bypass, cardiac thallium scan, exercise tolerance test, myocardial infarction or symptomatic/treated angina. Any individual who was asymptomatic or had negative investigations was categorised as having 'no presence of CHD'. Individuals suffering from the metabolic syndrome (MS) in UDACS were defined under the World Health Organisation classification [24] as exhibiting the following: a systolic BP greater than 130 mmHg and a diastolic BP over 80 mmHg; those on hypertensive therapy; triglyceride levels higher than 1.7 mmol/l; an HDL level lower than 1.0 mmol/l (1.29 mmol/l in females); a BMI greater than 30 kg/m^2; and all had clinically defined Diabetes. The UK Prospective Diabetes Study (UKPDS) risk algorithm was also applied to each participant at the beginning of the study and is comparable to that of the Framingham risk assessment, yet is specifically aimed at individuals with diabetes. The algorithm incorporates glycaemia, blood pressure, and lipid levels as risk factors in addition to age, sex, ethnic group, and smoking status [26].

The score was ranked out of 100, with the maximum rating being given to those people with a CHD event. Medical history was recorded and clinic measurements of blood pressure, weight, and height were measured on all subjects. Routine plasma traits were measured including plasma oxidised LDL by ELISA [27]. Oxidised LDL measures were then divided by total LDL in order to generate a specific measure of LDL oxidation [27]. All subjects were free from any acute illnesses at the time of recruitment.

2.3. Lp-PLA2 activity assay

Lp-PLA2 activity was measured, in duplicate, from citrated plasma stored at −80 °C by the trichloroacetic acid precipitation procedure in 96-well plates described previously [17]. The activity of Lp-PLA2 was expressed in nmol PAF hydrolysed/min per ml of plasma and the within assay variability was <5%. Lp-PLA2 activity was significantly lower than that found in other comparable studies despite being measured in the same laboratory [17,22]. Lp-PLA2 activity was measured in citrated plasma diluted by an estimated 15% due to a volume of citrate in each tube. When this correction was taken into account, the plasma activity levels were similar to those previously observed. This correction was not applied in subsequent analysis as it was based on an estimate only.

2.4. DNA extraction and PLA2G7 A379V genotyping

DNA was extracted using the salting out method [28]. *PLA2G7* A379V variant genotype (rs1051931) was determined for each subject by polymerase chain reaction (PCR) and restriction digest as described previously [21].

2.5. Statistical analysis

Departure from Hardy–Weinberg equilibrium was assessed using Chi-squared tests. All analyses were performed on normally distributed data after appropriate transformation (log or square root). A univariate step-wise regression model was subsequently applied to assess contributors to variance of Lp-PLA2 activity. Differences in continuous variables were examined using analysis of variance (ANOVA) and linear trend ($p<0.05$ was taken as statistically significant). Where adjustments were required, analysis of covariance was applied. For analysis of oxLDL/LDL by Lp-PLA2 genotype, women were included as there was no heterogeneity among sexes with regards to genotype effects on oxLDL/LDL.

3. Experimental results

Table 1 shows the baseline characteristics of the Caucasian subjects (men and women) in the UDAC study in those with or without CHD. As expected, those men who were positive for

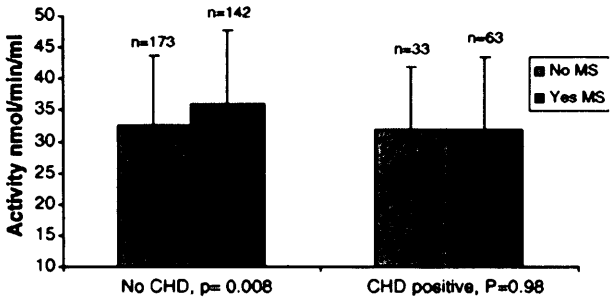

Fig. 1. Relationship between mean Lp-PLA2 activity (±1S.D.) and metabolic syndrome status. Those who had CHD were also characterised separately. Mean Lp-PLA2 activity was adjusted for type of diabetes and statin treatment.

CHD were older, more obese, had lower HDL, and higher triglycerides and CRP levels. They also showed a higher usage of statins and ACE inhibitors, potentially explaining their lower diastolic blood pressure, and LDL-C levels. Those women positive for CHD were older, and had a higher usage of statins, possibly explaining their lower LDL-C levels. Lp-PLA2 activity was significantly *lower* in those diabetic men with CHD (31.0 ± 10.5 nmol/min/ml) than those without (34.2 ± 11.3 nmol/min/ml, $p=0.005$). However, this was no longer significant after adjustment for age, BMI, total cholesterol, and drug usage (ACE inhibitors and statins) ($p=0.38$). There was no evidence that either type of diabetes or CHD status interacted with oxLDL/LDL, LDL-MPD/PPD, UKPDS risk score or metabolic syndrome (MS), in their effect on Lp-PLA2 activity. Type of diabetes, CHD status, and statin use were adjusted for in the following analysis.

3.1. Metabolic syndrome and UKPDS risk score in relation to Lp-PLA2 activity

Considering the relationship between those with features of the metabolic syndrome (MS) and Lp-PLA2 activity, those men with the MS ($n=205$) had a higher Lp-PLA2 activity (35.6 ± 11.9 nmol/min/ml) compared to those without the MS ($n=206$; 33.0 ± 10.8 nmol/min/ml) after adjustment for type of diabetes, CHD status and statin use ($p=0.02$). When considering those with CHD and those without separately (Fig. 1), the effect remained significant in those diagnosed as negative for CHD ($p=0.008$); however, there was no longer a significant difference in activity in those positive for CHD ($p=0.98$); both results were adjusted for type of diabetes and statin use. In addition, when the UKPDS risk algorithm [26] was divided into quartiles of risk and compared against Lp-PLA2 activity, there was a positive relationship between UKPDS CHD risk score and higher Lp-PLA2 activity ($p=0.006$ and $p=0.004$ for linear trend) (Table 2) when adjusted for CHD status, diabetes type, and statin use. This effect was borderline significant in those without CHD ($p=0.08$), while remaining significant in those positive for CHD ($p=0.01$) (Fig. 2).

Table 1
Baseline characteristics of the Caucasian subjects in the UDAC study by coronary heart disease status

	Males no CHD ($n=367$)	CHD ($n=107$)	p-Value	Females no CHD ($n=258$)	CHD ($n=51$)	p-Value
Age	59.3 (13.8)	67.5(11.2)	<0.0005	63.3 (14.1)	70.5 (8.4)	0.001
Type I diabetic (%)	29.2	4.8	<0.0005	23.6	5.9	0.002
BMI (kg/m^2)‡	28.1 (5.0)	29.3 (5.7)	0.03	28.4 (6.2)	29.6 (4.6)	0.19
Systolic BP (mmHg)‡	137.4 (18.6)	136.5 (23.8)	0.68	140.4 (20.5)	142.2 (17.5)	0.55
Diastolic BP (mmHg)‡	81.3 (11.3)	76.6 (12.5)	<0.0005	77.6 (10.4)	76.6 (9.8)	0.55
LDL (mmol/l)†	2.8 (0.8)	2.2 (0.6)	<0.0005	2.9 (0.7)	2.5 (0.7)	0.01
oxLDL (mU/l)†	46.3 (17.9)	45.3 (20.0)	0.67	48.7 (17.9)	47.6 (17.4)	0.75
oxLDL/LDL (U/mmol)*	16.9 (7.1)	19.3 (11.5)	0.04	16.7 (7.8)	18.4 (6.5)	0.25
HDL (mmol/l)‡	1.8 (0.6)	1.4 (0.4)	<0.0005	1.5 (0.5)	1.4 (0.4)	0.16
TG (mmol/l)‡	1.6 (1.0)	1.9 (1.1)	0.03	1.7 (1.0)	1.9 (0.9)	0.12
CRP (mg/l)‡	1.4 (1.2)	1.8 (1.5)	0.005	1.8 (1.5)	1.8 (1.7)	0.92
Statin use (%)	15	63	<0.0005	23	58	<0.0005
ACE I (%)	43	63	<0.0005	41	41	0.95
Lp-PLA2 activity (nmol/min/ml)*	34.2 (11.3)	31.0 (10.5)	0.005	na	na	na
Genotype AA	225(62.7%)	63(60.6%)	0.91	155 (63.5%)	29 (58.0%)	0.62
AV	123(34.3%)	38(36.5%)		76 (31.1%)	19 (38.0%)	
VV	11(3.1%)	3(2.9%)		13 (5.3%)	2 (4.0%)	
Rare Allele frequency	0.20	0.21		0.21	0.23	

* Antilog of log10 transformed mean. S.D. is approximate.
† Square of square root transformed mean. S.D. is approximate.
‡ Natural log geometric mean. S.D. is approximate.

3.2. Oxidised LDL/LDL measures and Lp-PLA2 activity

Oxidised LDL was not significantly different in those with or without CHD in men or women. However, the oxLDL level relative to LDL concentration (oxLDL/LDL) was significantly higher in those men with the presence of CHD (Table 1). Those men in the highest quartile of oxLDL/LDL level had the lowest Lp-PLA2 activity (31.3 ± 10.5 nmol/min/ml) when compared to the middle two (32.3 ± 9.8 and 35.9 ± 10.9 nmol/min/ml, respectively) and lowest quartile (35.6 ± 12.5 nmol/min/ml) ($p=0.03$ and $p=0.004$ linear trend) after adjustment for type of diabetes, CHD status, and statin use (Table 2). The effect was even more significant in those who did not have the presence of CHD ($p=0.008$), and was no longer seen in those with the presence of CHD ($p=0.37$) (Fig. 3).

3.3. Effect of A379V genotype on Lp-PLA2 activity and oxLDL/LDL

Although Lp-PLA2 activity was only measured in men, genotype data was available for both men and women. Table 3 shows the baseline characteristics of the UDAC study in men and women by A379V genotype. The genotype distribution did not depart from Hardy–Weinberg equilibrium ($p=0.431$). Since the 379V allele had previously shown

Table 2
Geometric mean for LpPLA2 in Caucasian men adjusted for diabetes type, CHD status and statin use

	N	Adjusted mean (±1S.D.)	Adjusted p-value
Metabolic syndrome			
No	206	33.0 (10.8)	0.02
Yes	205	35.6 (11.9)	
Oxidised LDL/LDL (U/mmol)			
Quartile 1 (0–13.6)	75	35.6 (12.5)	0.03
Quartile 2 (13.6–17.7)	66	35.9 (10.9)	0.004 linear trend
Quartile 3 (17.7–22.1)	65	32.3 (9.8)	
Quartile 4 (>22.1)	74	31.3 (10.5)	
UKPDS			
Quartile 1 (0–13.7)	69	33.4 (11.5)	0.006
Quartile 2 (13.7–22.4)	73	32.2 (11.2)	0.004 linear trend
Quartile 3 (22.4–36.7)	104	32.9 (10.5)	
Quartile 4 (>36.7)	127	37.4 (12.5)	
A379V genotype			
AA/AV	395	34.2 (11.4)	0.34
VV	12	37.5 (11.1)	

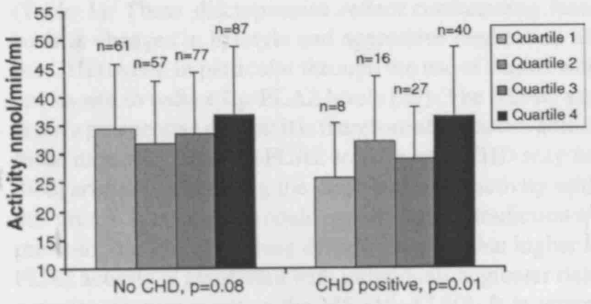

Fig. 2. Relationship between mean Lp-PLA2 activity (±1S.D.) and UKPDS risk quartiles in those men with CHD and those without. The range of UKPDS risk score is: quartile 1 (0–13.7), quartile 2 (13.7–22.4), quartile 3 (22.4–36.7), and the highest quartile 4 (>36.7).

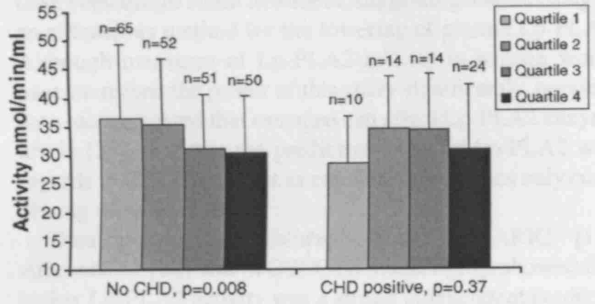

Fig. 3. Relationship between mean Lp-PLA2 activity (±1S.D.) and oxLDL/LDL quartiles in those men with CHD and those without. The range of oxLDL/LDL is: quartile 1 (0–13.6 U/mmol), quartile 2 (13.6–17.7 U/mmol), quartile 3 (17.7–22.1 U/mmol), and the highest quartile 4 (>22.1 U/mmol).

recessive effects [21], the AV and AA combined group was also compared to VV homozygotes. With regards to Lp-PLA2 activity, men homozygous for the 379V allele exhibited a non-significant trend towards higher Lp-PLA2 activity (37.5 ± 11.1 nmol/min/ml) compared to A379 carriers (34.2 ± 11.4 nmol/min/ml) ($p = 0.34$) (Table 2). A379V genotype showed no significant association with UKPDS risk score, and there was no significant difference in A379V allele frequency between those with features of the MS and those without (data not shown).

In male and female subjects, A379V genotype was not significantly associated with oxLDL/LDL ($p = 0.77$ and $p = 0.18$, respectively) (Table 3). Since there was no heterogeneity of effect of gender on oxLDL/LDL ($p = 0.85$), males and females were combined to examine this relationship further. In combined analysis A379V genotype was not associated with oxLDL/LDL levels ($p = 0.32$) (data not shown).

4. Discussion

In the UDAC study, men characterised as having CHD had *lower* Lp-PLA2 activity, as well as lower LDL levels and diastolic blood pressure, compared to those men without

Table 3
Baseline characteristics of Caucasian subjects in the UDAC study by A379V genotype

	Males AA ($n = 288$)	AV ($n = 161$)	VV ($n = 14$)	p-Value	p-Value A+/VV	Females AA ($n = 184$)	AV ($n = 95$)	VV ($n = 15$)	p-Value	p-Value A+/VV
Age	61.0 (13.5)	61.6 (13.9)	60.0 (15.1)	0.87	0.75	63.1 (14.2)	67.1 (12.2)	66.9 (9.0)	0.05	0.49
HDL (mmol/l)‡	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)	0.99	0.91	1.5 (0.5)	1.5 (0.4)	1.4 (0.4)	0.75	0.51
LDL (mmol/l)†	2.6 (0.7)	2.5 (0.8)	2.8 (0.9)	0.46	0.31	2.8 (0.8)	2.7 (0.6)	2.9 (0.7)	0.39	0.78
oxLDL (mU/l)†	44.3 (18.4)	43.8(18.7)	49.6 (17.4)	0.68	0.39	48.0 (21.6)	45.5 (19.6)	41.6 (14.8)	0.18	0.21
oxLDL/LDL (U/mmol)*	17.5 (7.7)	17.3 (9.3)	15.5 (5.2)	0.77	0.49	16.7 (7.5)	17.5 (7.5)	14.0 (5.0)	0.19	0.18
Lp-PLA2 activity (nmol/min/ml)*	33.8 (10.9)	32.4 (11.7)	36.9 (11.1)	0.25	0.28	na	na	na	na	na

Due to the recessive nature of the A379V polymorphism, A379 allele carriers were also combined together.
* Antilog of log transformed mean. S.D. is approximate.
† Square of square root transformed mean. S.D. is approximate.
‡ Natural log geometric mean. S.D. is approximate.

(Table 1). These discrepancies reflect confounding factors such as changes in lifestyle and aggressive medication after the CHD event, in particular through the use of statins which are known to reduce Lp-PLA2 levels [29]. The UDAC study is not a prospective design; it is therefore also conceivable that those men with high-Lp-PLA2 activity with CHD may have died, artificially lowering the mean observed activity within this group. These factors could explain the contradiction with previous studies of differing design, showing that higher Lp-PLA2 activity is associated with individuals at greater risk of a cardiovascular event or the MS [15–17,30]. It is interesting to note that the observed reduction in Lp-PLA2 activity in those individuals positive for CHD was removed after adjustment for drug treatment and changes in lipid parameters. This would suggest that the difference in activity seen could indeed have been due to statin treatment, suggesting that this may be an efficacious method for the lowering of plasma Lp-PLA2. Although measures of Lp-PLA2 activity in women would have increased the power of this study significantly, previous data has suggested that estrogen can affect Lp-PLA2 enzyme levels [25], and that the predictive value of Lp-PLA2 with regards to CHD risk is not as conclusive in studies only comprising of women [16].

Data previously published from the ARIC [15], Athero*Gene* [22] and WOSCOPS studies [18], showed that higher Lp-PLA2 activity was a strong independent predictor of cardiovascular disease. Although the UDAC study is cross-sectional in design and has limited power to investigate risk using odds ratios; the UKPDS CHD risk algorithm and MS definition allowed the association of Lp-PLA2 activity and risk to be investigated. Those individuals with features of MS are at a higher risk of developing atherosclerosis [reviewed in [31]]. In UDACS, Lp-PLA2 activity was 7.9% higher in men with the MS compared to those without, although in those patients with the presence of CHD this difference in activity was not seen, possibly due to the aggressive drug therapy and lifestyle changes implemented post-event. The significant positive association of UKPDS risk score with Lp-PLA2 activity supported the observations regarding the MS, and suggests that Lp-PLA2 activity acts as an accurate predictor or marker of CHD risk (as defined by UKPDS and MS) in diabetic subjects. In comparison to PROCAM and FRAMINGHAM risk scorings, the UKPDS risk algorithm was based upon a study of patients with diabetes (5102 patients who were followed for just over 10 years) [26] and has previously been found to be a comparable risk predictor to those scoring techniques [32].

The association of Lp-PLA2 activity with oxidised LDL raises interesting questions about the potential pro- or anti-atherosclerotic roles of this enzyme. Higher oxidised LDL/LDL levels were significantly associated with lower Lp-PLA2 activity in the UDAC study, although this association was not apparent in those individuals with the presence of CHD, again suggesting that treatment has dampened an intermediate phenotype associated with the progression of atherosclerosis. The oxLDL/LDL measure was used to correct for differences in LDL concentration and sheds light on processes governing the rate of LDL particle oxidation [27]. This association supports previous data showing that Lp-PLA2 protected LDL in vitro from lipid peroxidation, with the removal of this enzyme leading to enhanced oxidation as measured by conjugated dienes [33]. If Lp-PLA2 is removing pro-inflammatory oxidised phospholipids and PAF, then the contradicting association between Lp-PLA2 activity and risk suggests that this is a consequence of CHD, and not causal of it. However, the hydrolysing of these molecules could, in turn, generate high levels of lyso-PC and free fatty acids, making the enzyme pro-atherogenic. In which case, the observed association between activity and risk seen here represents a causal relationship of this enzyme with atherosclerosis.

With regards to *PLA2G7* genotype, there was no significant effect of the A379V polymorphism on Lp-PLA2 activity in the UDAC study. Those individuals homozygous for the 379V allele had 9.6% higher Lp-PLA2 activity compared to A379 carriers, although this difference did not reach statistical significance ($p = 0.34$). This data supports results from the Athero*Gene* study, which showed a weak but significant association between the 379V variant and *higher* activity [22]. Since the *PLA2G7* 379V allele has been previously associated with a lower risk in the HIFMECH and Athero*Gene* studies [21,22], this suggests that elevated Lp-PLA2 activity may be important in the *prevention* of atherosclerosis. However, in vitro [20] the 379V Lp-PLA2 enzyme hydrolysed labelled-PAF at an increased K_m and V_{max} compared to the A379 form of the enzyme (although the K_m and V_{max} values were obtained with recombinant *E. coli* enzyme which was neither glycosylated or attached to lipoproteins). In the two published studies to date, the 379V allele appears to be protective [21,22]. This brings into question the exact relationship between Lp-PLA2 activity, *PLA2G7* A379V genotype and risk. It is clear that further in vitro and in vivo work is needed to establish the precise nature of the altered enzyme kinetics of the A379V mutation on Lp-PLA2 function.

The UDAC study failed to identify a statistically significant effect of A379V genotype on Lp-PLA2 activity, oxLDL/LDL levels or risk (as defined by MS and UKPDS). With contradicting data concerning the A379V polymorphism and Lp-PLA2 activity, it is difficult to make any conclusions as to the functionality of this SNP. The UDAC study may not have been powered sufficiently to investigate these associations. Further functional work is needed in order to determine the exact functional effect of the A379V polymorphism on Lp-PLA2 activity, since the mutation could not only influence overall activity, but also the specificity of this enzyme for certain phospholipid substrates.

There is also the possibility that Lp-PLA2 could be exerting pro- and anti-atherogenic effects depending on the in vivo location of the enzyme. Higher levels of Lp-PLA2 activity in the plasma could prevent atherosclerosis with the increased removal of pro-inflammatory PAF,

and oxidised phospholipids on the surface of LDL particles [34]. The generation of lyso-phospholipids and free fatty acids, although contributing to a pro-inflammatory status, may not reach concentrations in the plasma that are physiologically important. However, once in the arterial wall, Lp-PLA2 may generate a physiologically important concentration of lyso-phospholipids and free fatty acids, altering Lp-PLA2 from a beneficial enzyme to one that is detrimental. However, until definitive evidence about the role of A379V polymorphism on activity or substrate preference becomes clear, these data must be interpreted with caution.

Only two studies to date, Athero*Gene* and UDACS, have considered the relationship between *PLA2G7* genotype and Lp-PLA2 activity, and only the Athero*Gene* and HIFMECH studies have examined and found a positive relationship between genotype to risk [21,22]. The appropriate way to further examine the relationship between Lp-PLA2 and CHD is by the use of mendelian randomisation [35]. A test of causality could be achieved by studying the relationship between CHD risk and a genetic determinant (A379V genotype) of an intermediate phenotype (in this case Lp-PLA2 activity). Since alleles are allocated essentially at random at conception, such an association would not be subject to either confounding or reverse causation. If a causal relationship between *PLA2G7* genotype and activity were clearly established, then an association between *PLA2G7* genotype and CHD risk would provide indirect evidence for the causality of the association between Lp-PLA2 activity and CHD risk. There is a clear need for studies large enough to resolve the relationship between *PLA2G7* A379V polymorphism, Lp-PLA2 activity and CHD risk.

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Lipoprotein-associated phospholipase A2 A379V variant is associated with body composition changes in response to exercise training

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KEYWORDS
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Abstract Lipoprotein-associated PLA2 (Lp-PLA2) hydrolyses the *sn*-2 position of glycerophospholipids, in particular platelet activating factor (PAF), generating significant amounts of Lyso-PAF which in turn, via a remodelling pathway, can generate arachidonic acid (AA) from alkyl-acyl-glycerophosphorylcholine. AA is a precursor for prostaglandin synthesis, which regulates adipogenesis through the peroxisome proliferator-activated receptor subfamily. AA may also modulate skeletal muscle growth. We investigated the association of the *PLA2G7* A379V variant with changes in body composition in a longitudinal study of 123 male Caucasian army recruits over 10 weeks of intensive physical training. There was no effect of genotype on baseline measures. However, after exercise training, homozygosity for the 379V allele was associated with a *decrease* in percentage adipose tissue mass ($-3.61 \pm 1.14\%$), compared to AV ($-1.67 \pm 0.38\%$) and AA ($-1.09 \pm 0.24\%$) genotypes ($p = 0.01$), and a significant mean *increase* ($3.51 \pm 1.17\%$) in percentage lean mass, compared to AV ($1.64 \pm 0.38\%$) and AA ($1.10 \pm 0.24\%$) recruits ($p = 0.02$). The association of this genotype with changes in body composition after training suggests a novel role for Lp-PLA2.

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Background and aims

Lipoprotein-associated PLA2 (Lp-PLA2) or platelet activating factor acetylhydrolase (PAFAH) is a member of the super-family of enzymes defined by their ability to hydrolyse the middle (*sn*-2) ester bond of phospholipids [1]. The Lp-PLA2 gene (*PLA2G7*) maps to chromosome 6p 12–21.1, and encodes a 45-kDa protein [2]. Lp-PLA2 is secreted into the plasma by platelets, neutrophils, macrophages, T-lymphocytes [3], and tissue sources that are consistent with the macrophage origin of the enzyme (e.g. the thymus and tonsil) [4]. Approximately 70–80% of the plasma enzymatic activity of Lp-PLA2 is associated with low density lipoprotein (LDL) particles, with the remainder residing on high density lipoprotein (HDL) [3,5,6].

Lp-PLA2 has a marked substrate preference for glycerophospholipids including platelet activating factor (PAF), and short fatty acid chain oxidised phospholipids, both of which interact with a cell surface G-protein-coupled receptor, leading to inflammatory cell activation [7]. In this context, Lp-PLA2 may play an *anti-atherogenic* role by converting a potent inflammatory signalling molecule present in plasma into two biologically inactive metabolites, Lyso-PAF and acetate [8].

In contrast, recent evidence suggests that Lp-PLA2 may also have *pro-inflammatory* activity associated with the generation of significant amounts of Lyso-phosphatidylcholine (Lyso-PC) and free fatty acids from the degradation of oxidised phosphatidylcholine (PC) [9]. The association of Lp-PLA2 with LDL, via an interaction with apolipoprotein (apo)B [10], makes this enzyme ideally placed to participate in the key oxidative steps of atherogenesis, with Lyso-PC and oxidised free fatty acids being largely responsible for the pro-atherogenic activity attributed to oxidised LDL [11]. In addition to this, Lp-PLA2 and other phospholipase A2 enzyme (PLA2) family members are involved in the remodelling of platelet activating factor (PAF) to Lyso-PAF, and the consequent release of arachidonic acid by the generation of alkyl-acyl-glycerophosphorylcholine from Lyso-PAF [12,13]. Arachidonic acid (AA) is a precursor of the eicosanoid family of potent inflammatory mediators that includes prostaglandins, thromboxanes, leukotrienes, and lipoxins. Prostaglandin I_2 (prostacyclin), which is also synthesised and released from pre-adipocytes, has previously been identified as one of the main adipogenic components of serum [14]. PLA2 remodelling of PAF is a significant pathway through which AA is liberated from phospholipids, and may therefore play a central role in a diverse range of signalling pathways, one of which leads to adipocyte differentiation and hence alterations in adipose tissue mass [15].

Several variants within the Lp-PLA2 gene (*PLA2G7*) have previously been used in association studies to examine a possible relationship to coronary heart disease (CHD) risk and asthma [16,17]. We recently reported an association between the A379V polymorphism and CHD risk in a case–control study, with V379 homozygotes being protected from CHD [18]. This was supported by results from AtheroGene, showing that those individuals homozygous for the 379V allele were associated with a reduced risk of coronary artery disease (CAD) [19]. In vitro analysis of the A379V variant in a recombinant enzyme, showed a two-fold increase in the K_m of the V379-Lp-PLA2 enzyme for PAF [17]. The association of this genotype with lower Lp-PLA2 substrate affinity may support a pro-inflammatory causative role for Lp-PLA2 in CHD.

Due to the potential modulating effect of Lp-PLA2 on AA release, we have examined the association of *PLA2G7* A379V genotype with body composition change in the Bassingbourn Army Study II, a study of young healthy Caucasian male recruits over a 10-week intensive exercise training period [20]. The body mass/composition response of such subjects to training, and the fact that this is genetically influenced, has already been described in detail [21].

Methods

Study group

The study group comprised of 144 Caucasian male recruits, aged between 16 and 22 years, drawn from the army training regiment, Bassingbourn, UK, over an 18-month period [20]. All were normotensive and free from overt cardiovascular disease, and underwent an identical 10-week intensive physical exercise programme involving mixed strength and endurance training conducted by the British Army. The study was aimed to examine the effect of the angiotensin II type I receptor antagonist, Losartan, on left ventricular growth, and to investigate the association of angiotensin converting enzyme (*ACE*) gene variation with exercise-induced left ventricular growth. Recruits were genotyped for the *ACE* insertion (I)/deletion (D) polymorphism and those homozygous for either the *ACE* I or D alleles were invited to participate in the trial, and written informed consent was obtained. Subjects were studied at the beginning and end of the

training period, at which time, weight and blood pressure (mean of three manual measurements) were recorded. In addition to recruits undergoing cardiac magnetic resonance (CMR) to determine LV mass, 40 10-mm thick MRI images of the whole body were obtained, adipose tissue mass was quantified, and lean mass was calculated by subtracting adipose tissue mass from total body mass [20]. After baseline scans, each II or DD group was independently randomised (conforming to a prospective parallel arm double blind, randomised controlled trial protocol) to receive either 25 mg Losartan or placebo daily (compliance was monitored thoroughly by officers). The Defence Medical Services College Research Committee (DMSCR) approved the human subjects protocol described.

Genotyping

PLA2G7 A379V variant genotype was determined for each subject by polymerase chain reaction (PCR) using a sense primer (5′-AGGGAGACATAGATTCAACTG-3′) and anti-sense primer (5′-CGTTTTGTAAGAATGCTAATGAA-3′). Use of these primers introduces a *PstI* site in the presence of the A379 allele. The size of the undigested fragment is 69 bp and is restricted to 49 bp and 20 bp by *PstI*. Restriction enzyme digestion was performed in a volume of 13 μl containing 5 μl of the PCR product and the buffer recommended by the manufacturer (Promega Inc.) for 4 h at 37 °C. Fragments were resolved using Microtitre Array Diagonal Gel Electrophoresis on an ethidium-bromide stain, 7.5% polyacrylamide gel [22]. One heterozygote individual (as confirmed by sequencing) and one negative control were included in each PCR run. All rare homozygote samples were re-amplified and re-digested to confirm genotype.

Statistical analysis

Departure from Hardy–Weinberg equilibrium was assessed using Chi-squared tests. Differences in continuous variables were examined using analysis of variance (ANOVA) and linear trend ($p < 0.05$ was taken as statistically significant). All the variables analysed were normally distributed. For studying alterations in lean mass and adipose tissue, the 'percentage change' measure takes into account baseline differences in body mass, and was determined in the following way:

$$\text{Percentage change in lean or adipose tissue mass} = \frac{\text{mass},x - \text{mass},y}{\text{mass},y} \times 100$$

where x = post-training and y = pre-training.

Results

One hundred and forty-four young healthy British Army recruits, randomised to receive either low-dose Losartan or placebo, were genotyped for the *PLA2G7* A379V variant. A379V genotype distribution was in Hardy–Weinberg equilibrium with a rare allele frequency of 0.22 (95% CI 0.16–0.27), which was similar to reported frequencies in European subjects in the HIFMECH study and Caucasian patients with asthma [17,18]. There was no heterogeneity of any of the qualitative traits related to Losartan treatment [20], and *ACE* genotype was also found not to significantly affect lean mass and adipose tissue mass change (data not shown), therefore analysis was performed on the group as a whole.

Baseline characteristics for the group are shown in Table 1. Age, body mass index (BMI), diastolic/systolic blood pressure, lean mass, and adipose tissue did not differ significantly among genotype groups.

After 10 weeks of intensive training, there was no statistically significant difference in left ventricular mass (LVM) and systolic blood pressure by A379V genotype. VV homozygotes showed a trend, although not statistically significant, towards a loss in weight whereas the AA and AV genotype groups gained weight over the 10-week training period (Table 2 and Fig. 1). When the changes in body

Table 1 Baseline characteristics of recruits by genotype (mean ± SE)

Parameters	AA ($n = 77$)	AV ($n = 39$)	VV ($n = 7$)	p (ANOVA)
Age (y)	19.52 ± 0.29	19.83 ± 0.44	20.50 ± 0.24	0.66
BMI (kg/m^2)	23.13 ± 0.23	23.08 ± 0.34	23.07 ± 1.14	0.99
Diastolic BP (mm Hg)	66.28 ± 1.20	66.49 ± 1.71	63.80 ± 2.63	0.87
Systolic BP (mm Hg)	118.05 ± 1.40	117.83 ± 1.74	113.60 ± 3.50	0.72
Adipose tissue mass (kg)	12.08 ± 0.44	12.10 ± 0.57	14.62 ± 2.03	0.30
Lean mass (kg)	59.57 ± 0.65	57.14 ± 1.07	58.55 ± 1.42	0.13

Table 2 Change in variables over the 10-week training period by genotype (mean ± SE)

Parameters	AA ($n = 77$)	AV ($n = 39$)	VV ($n = 7$)	p (ANOVA)	p (Linearity)
Systolic BP change (mm Hg)	0.90 ± 1.47	1.09 ± 1.09	−1.33 ± 6.20	0.92	0.86
Diastolic BP change (mm Hg)	−0.50 ± 1.31	0.22 ± 1.77	1.67 ± 5.81	0.89	0.64
%LV mass change	4.53 ± 0.80	6.31 ± 1.18	2.73 ± 4.12	0.37	0.59
%Change in weight	0.78 ± 0.45	1.79 ± 0.62	−1.40 ± 2.14	0.13	0.96
Lean mass change (kg)	1.22 ± 0.19	2.09 ± 0.35	1.70 ± 0.62	0.06	0.05
Adipose tissue mass change (kg)	−0.82 ± 0.21	−0.89 ± 0.28	−3.14 ± 1.15	0.01	0.03
Percentage lean mass change (%)	1.10 ± 0.24	1.64 ± 0.38	3.51 ± 1.17	0.02	0.01
Percentage adipose tissue change (%)	−1.09 ± 0.24	−1.67 ± 0.38	−3.61 ± 1.14	0.01	0.01

composition over the training period were examined, recruits homozygous for the V379 allele showed a significant decrease in adipose tissue mass over the 10-week training programme (−3.14 ± 1.15 kg) when compared to AV (−0.89 ± 0.28 kg) and AA (−0.82 ± 0.21 kg) genotype groups ($p = 0.01$) (Table 2 and Fig. 2). The AV genotype group showed a significant increase in lean mass (2.09 ± 0.35 kg) compared to A379 homozygotes (1.22 ± 0.19 kg, $p = 0.02$) (Table 2 and Fig. 2). The change in lean muscle mass and adipose tissue was found to be tightly negatively correlated in this study ($r = -0.91$, $p < 0.01$).

When corrected for baseline differences in body size among recruits by analysing the changes in percentage adipose tissue and lean mass, individuals homozygous for the 379V allele showed a significant decrease in percentage adipose tissue (−3.61 ± 1.14) compared to AV (−1.67 ± 0.38) and AA (−1.09 ± 0.24) genotype groups ($p = 0.01$, ANOVA and linear trend) (Table 2 and Fig. 3). There was also a significant increase in percentage lean mass in VV recruits (3.51 ± 1.17) compared to AV (1.64 ± 0.38) and AA individuals (1.10 ± 0.24, $p = 0.02$ for ANOVA and $p = 0.01$ for linear trend) (Table 2 and Fig. 3). When AA individuals are compared to those carriers of the V allele (77 and 46 individuals, respectively), the association of the 379V allele with percentage lean mass and percentage adipose tissue changes was still significant ($p = 0.05$, data not shown). Adjustment for potential confounders such as age, systolic blood pressure, *ACE* I/D genotype, and Losartan treatment did not significantly affect the observed results.

Discussion

Defining the role of Lp-PLA2 in cardiovascular disease is challenging due to the opposing pro- and anti-atherosclerotic actions attributed to the enzyme. The anti-inflammatory degradation of PAF or PAF-like oxidised phospholipids, and thus the removal of an important inflammatory signalling molecule is well documented [4,23,24]. However, Lp-PLA2 may also promote inflammation via the production of Lyso-phospholipids and free fatty acids at high concentrations in the intima of arteries [25,26]. These molecules are potent mediators of inflammation and cell signalling, and it has

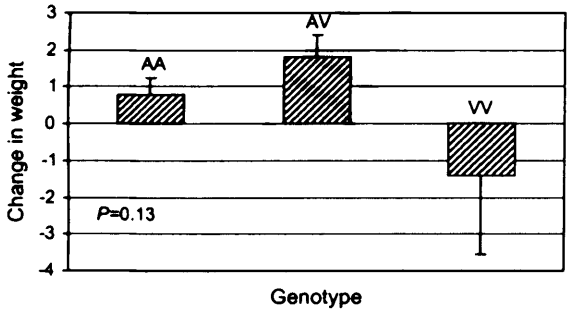

Figure 1 Graph showing the mean change (±SE) in weight over a 10-week training period stratified by *PLA2G7* A379V genotype.

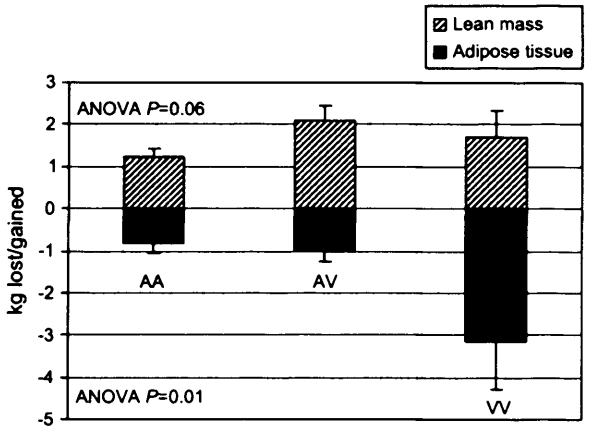

Figure 2 Graph showing the mean change in lean and adipose tissue mass in kg (±SE) over the 10-week training period stratified by A379V genotype.

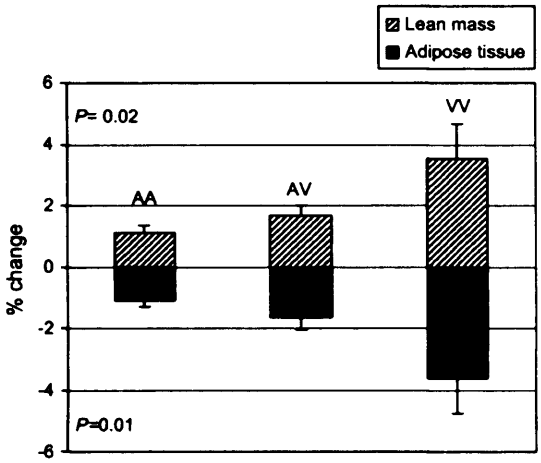

Figure 3 Mean percentage change in lean and adipose tissue mass (±SE) over the 10-week training period stratified by A379V genotype.

been suggested that high levels of Lp-PLA2 may increase risk of cardiovascular disease [9,27,28].

In this study we examined the A379V variant of Lp-PLA2, where the V379 allele is associated with a two-fold lower Lp-PLA2 affinity for PAF [17]. This may be due to the proximity of the A379 to the H351 residue of the Lp-PLA2 amino acid sequence, which together with S273 and D296 forms a catalytic triad critical for enzyme activity [29]. However, the A379V polymorphism may lead to, as yet unspecified, subtle changes in substrate specificity rather than an overall change in activity. Indeed, recent observations in the AtheroGene study showed that those individuals homozygous for the 379V allele were associated with a weak but significantly higher activity, contrasting with the observations made by Kruse et al. [17] in vitro. Although there was no significant effect of genotype on overall change in weight over the 10-week training period, there was a strong association between A379V genotype and changes in body composition. V379 homozygotes were associated with a significant *lowering* in percentage adipose tissue mass, whilst also associating with a significant *increase* in percentage lean mass compared to AV and AA individuals. The significant linear trend with AV recruits exhibiting intermediate values suggests that even though the numbers of 379V homozygotes were small, the conclusions are still valid.

In a sepsis model it has been shown that serum Lp-PLA2 levels correlate significantly with several cascade substances downstream of AA production, such as leukotriene B4 and thromboxane B2 [30]. In addition, recently published data have shown that PAF initiates the release of AA in a murine macrophage cell line, potentially via the PAF receptor and eventual activation of cytosolic PLA2 [31]. The generation of AA and eicosanoids may affect adipose tissue differentiation by the activation of peroxisome proliferator-activated receptors (PPAR), which mediate the effects of fatty acids and their derivatives at the transcriptional level. There is evidence that long chain fatty acids such as AA and a number of derivatives are ligands for PPARγ [32–35], a critical regulator of adipocyte differentiation and function [36]. Whilst the precise mechanism of PPARγ activation by this pathway is under debate, it is thought that prostacyclin binds with its cell surface receptor, activating the protein kinase A pathway [37], and up-regulating the early expression of two transcription factor CCAAT-enhancer binding proteins (C/EBPδ and C/EBPβ) [38]. Secondly, there is evidence that prostacyclin binds directly with PPARδ [32]. C/EBPδ, C/EBPβ, and PPARδ and then act together in pre-adipocytes to upregulate the critical expression of PPARγ, leading to adipogenesis [14].

Higher Lp-PLA2 activity has previously been found to be associated with obese, diabetic individuals [39]. A possible mechanism for the effects seen here is that the A379V polymorphism is associated with differences in Lp-PLA2 activity [17], which could in turn affect the hydrolysis of PAF to Lyso-PAF, eventually altering the concentration of AA and its derivatives through this remodelling pathway [12]. Variation in AA could affect PPARγ activation and pre-adipocyte differentiation, and thus fat mass. This effect may be amplified by the fact that intense exercise can induce an inflammatory response [40], with Lp-PLA2 and prostaglandins being known to be elevated under these circumstances [41,42]. The A379V polymorphism may exhibit effects on substrate specificity that have not yet been fully elucidated, but could still influence the rate of AA production in vivo. The related secretory PLA2 enzyme (Type IIa) has also been shown to be correlated with body mass index [43], and the release of AA upon hydrolysis of phospholipids [44], supporting the mechanism by which PLA2 enzymes affect body composition through this remodelling pathway.

Apart from the synthesis of AA, another important cell signalling molecule produced by the remodelling pathway and potentially implicated in the modulation of adipocyte differentiation is LPA. Lp-PLA2 and other secretory PLA2 enzymes are able to hydrolyse oxidised acyl chain phospholipids, generating Lyso-PC. Lyso-PC and Lyso-PAF are substrates for lysophospholipase D leading to the generation of LPA [12]. Lysophosphatidic acid acts through its specific G-protein-coupled receptors which trigger proliferation, migration, and

survival in a variety of cells. LPA is also synthesised at the extracellular face of adipocytes by a secreted lysophospholipase D [45]. LPA present in conditioned media increases the growth of preadipose cell lines in culture through the activation of MAPK [46], however, recent in vitro work suggests that LPA exhibits an inhibitory effect on the development of adipose tissue, through the inhibition of PPARγ expression [45]. Variation in the activity of Lp-PLA2 (associated with the A379V variant) could therefore influence this pathway as well, explaining some of the effects seen regarding adipose tissue mass changes in the BH2 study.

The results obtained here to identify an association of Lp-PLA2 with body composition change with exercise, and this may in turn represent a new mechanism by which Lp-PLA2 may affect an individual risk of CVD. Obesity is associated with metabolic disorders such as dyslipidemia, diabetes, hypertension, and the metabolic syndrome and fat mass excess in severe obesity are typically due to an increase in both adipocyte size and number [14].

The change in percentage lean mass observed is more difficult to fully explain with our current understanding. Metabolites from the generation of AA regulate muscle-protein turnover, in particular elevated levels of Prostaglandin E2, have been implicated in cancer cachexia [47]. Cytosolic PLA2 has also been identified as a negative modulator of striated muscle growth. *Pla2g4a−/−* mice have exaggerated skeletal and cardiac muscle growth, corrected by the addition of AA [48].

In conclusion, we report for the first time that Lp-PLA2 genotype is associated with body composition in healthy young males exposed to prolonged rigorous exercise. Unfortunately, the small sample size of this study has meant that the VV homozygous group comprises only seven individuals, although the study design of shared environment, identical training, and accurate phenotypic measurement at baseline and follow-up increases the power of the study considerably. In addition, no measures of Lp-PLA2 activity or arachidonic acid were available to assess the effects of A379V genotype on these traits. Despite limitations, this study proposes a novel role for PLA2 in the development of obesity, and by its association cardiovascular disease.

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